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A COMPARATIVE ELECTROPHORETIC STUDY OF AN ALLOPOLYPLOID COMPLEX IN *BRASSICA*

by

Ⓒ MICHAEL B. COULTHART

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submitted by Michael B. Coulthart

in partial fulfilment of the requirements for the degree of Master of Science in Plant Chemotaxonomy.

Supervisor

James M Mayo

Curtis Strobeck

Date July 23, 1979

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ABSTRACT

A comparative electrophoretic study was undertaken in an alloploid complex in the genus *Brassica*. The material belonged to two groups of taxa. One group consisted of the natural allotetraploid *B. napus* and two diploid representatives of its parental species, *B. campestris* and *B. oleracea*. The other group consisted of two artificially doubled autotetraploid stocks (one of *B. campestris* and one of *B. oleracea*), a synthetic allotetraploid derived from a cross between these, and two diploid stocks corresponding taxonomically to the autotetraploids.

The feasibility of zymogram analysis of individual plants by disc electrophoresis was established for 14 leaf enzymes. Of these, 5 were examined on slab gels to determine zymogram phenotype variation and relationships within and between taxa of the two groups. From the slab gel data two putative structural gene loci, *Pgm-l* and *Sod-l*, were identified for phosphoglucomutase and superoxide dismutase respectively.

Analysis of electrophoretically variant alleles at these two loci permitted the following conclusions: (1) Both parental *Pgm-l* loci are expressed in the allotetraploids, and alleles found in their progenitors fully explained the zymogram-phenotypic variation in each; (2) The chromosome carrying the *Sod-l* locus in *B. campestris* may be present in tetrasomic dosage.

The results from the 3 other enzymes run on slab gels allowed no precise genetic interpretation. However, variants of glutamate dehydrogenase may have potential for the study of multilocus control of enzyme structure, and glutamate-oxaloacetate transaminase allozymes should prove very useful as a model system for the evolution of hybrid isozymes in allopolyploids.

It is concluded that the investigation of genome structure in diploids and genome expression in allopolyploids of *Brassica* using electrophoresis is practicable. The value of genetic information in chemotaxonomy is also discussed.

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INTRODUCTION

The genus *Brassica* (Cruciferae) consists of some one hundred species, a number of which are crops of major importance. Table 1 lists the six closely related members of the group to which most of these economic species belong.

Table 1

Species	Gametic Chromosome Number (n)
Brassica nigra Koch	8
Brassica oleracea L.	9
Brassica campestris L.	10
Brassica carinata Braun	17
Brassica juncea Coss.	18
Brassica napus L.	19

The phylogenetic relationships of these six species, originally suggested by the work of Morinaga (1934), were elucidated by U (1935). The three species with higher chromosome numbers (n=17, 18, 19) were shown to have originated by hybridization and polyploidy ("alloploidy", Stebbins 1947) from the three species with lower numbers (n=8, 9, 10). Figure 1 illustrates these relationships. For clarity, the haploid genomes of *B. campestris, B. nigra* and *B. oleracea* were termed the *a*, *b* and *c* genomes respectively.

U used the experimental cytological method of "genome analysis", which is useful for the determination of genomic affinities of polyploid species. In this procedure, meiotic pairing of chromosomes is observed

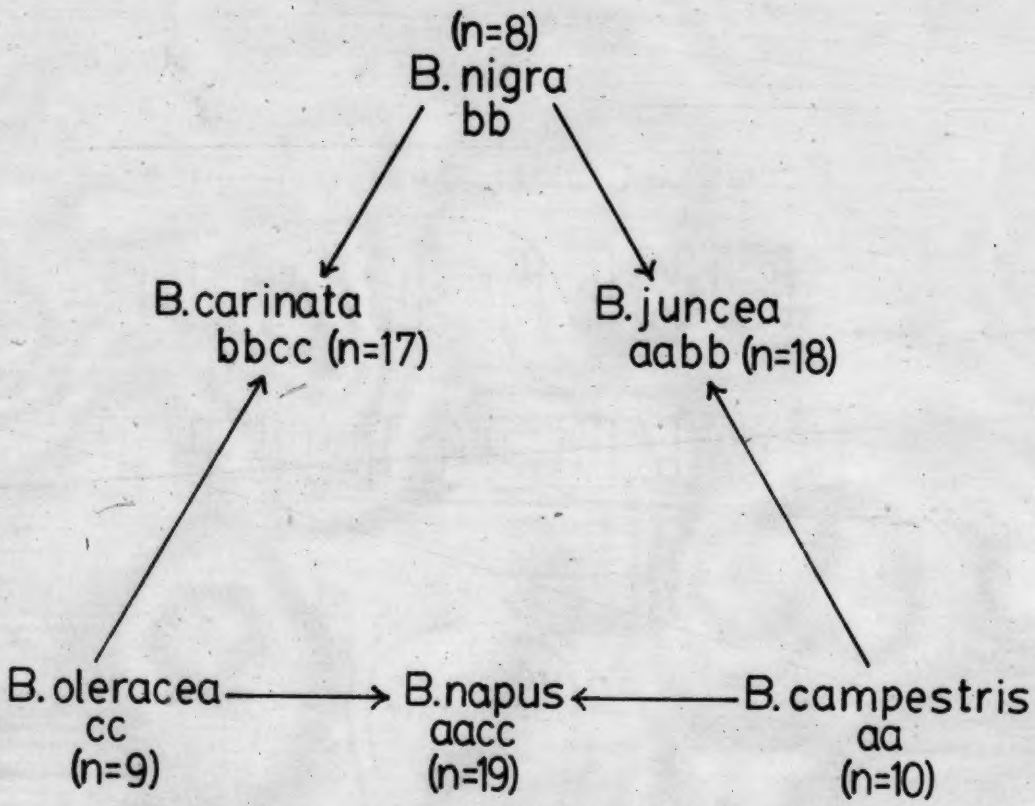

(n=8)
B. nigra
bb
B.carinata
bbcc (n=17)
B.juncea
aabb (n=18)
B. oleracea
cc
(n=9)
B.napus
aacc
(n=19)
B.campestris
aa
(n=10)

Figure 1. Genome relationships between diploid and allotetraploid species of *Brassica*. Gametic chromosome numbers are indicated in parentheses. Somatic genome compositions are represented by the letters a,b,c. Arrows indicate hybridization plus doubling of chromosome number.

in interspecific hybrids, on the assumption that the ability of chromosomes to form synaptic associations during meiotic prophase reflects similar linear arrangements of genes along their lengths. If this kind of analysis succeeds in implicating certain species as likely progenitors of the polyploid species in question, excellent evidence for the correctness or incorrectness of such conclusions can be obtained from an attempt to resynthesize the polyploid from the putative progenitors, using controlled crosses and/or various chromosome doubling techniques. This type of resynthesis has been achieved repeatedly for *Brassica carinata* (Frandsen 1947; Mizushima 1950; Mizushima and Katsuo 1953), *B. juncea* (Frandsen 1943; Olsson 1947, 1960a; Ramanujam and Srinivasachar 1943), and *B. napus* (Frandsen 1947; Hoffman and Peters 1968; Koch and Peters; 1953; Olsson 1953; Olsson *et al.* 1955; Rudorf 1950; U 1935). In all cases, the artificial amphiploid closely resembled the natural form, with artificial *B. napus* and *B. juncea* sometimes being difficult to distinguish from natural *B. napus* and *B. juncea* (Olsson 1960a, b).

In spite of the extensive documentation on this level of genome relationships within the group, structural relationships between the *a*, *b* and *c* genomes of *Brassica* remain incompletely clarified. Detailed karyotypes based on conventional morphological features visible in mitotic preparations have not been forthcoming, mainly due to the small size and resistance to staining of chromosomes. Furthermore, meiotic pairing in hybrids provides only ambiguous information on details of structural homology, especially with the prevalence in the genus of so-called "secondary association" between chromosomes belonging to the same genome (Kamala 1976a; Nwankiti 1970), and the generally low chiasma frequency found even in non-hybrids (Wills 1966). Furthermore, crosses between *B. campestris, B. nigra* and *B. oleracea* are quite difficult to make.

However, various types of indirect evidence have suggested general features of structural relationship between the genomes. Several early workers concluded that *B. nigra, B. oleracea* and *B. campestris* are of polyploid origin and that chromosome losses from an unknown ancestor with n=10 or n=12 account for the aneuploid series in the three species (Alam 1936; Catcheside 1934, 1937; Haga 1938; Ramanujam 1941; Richharia 1937; Reviews in Sikka 1940 and Yarnell 1956). Their conclusions were based mostly on observations of chromosome pairing in haploids, hybrids and normal types, and of multisomic segregation ratios in these supposed diploids. More recent investigators have adduced additional evidence of the same general nature in favour of polyploidy, and where sufficient data were available have proposed a base number of 6 (Kamala 1976a, b, 1978; Nwankiti 1970; Prakash 1973a, b, 1974; Röbbelen 1960; Venkateswarlu and Kamala 1971; Wills 1966). In certain cases, studies of morphology and association of pachytene chromosomes (Kamala 1976a; Röbbelen 1960; Venkateswarlu and Kamala 1971) have even allowed the postulation of chromosome formulas for the three genomes: *a*= AA B C DD E FFF, *b*= A B C DD EE F, and *c*= A BB CC D EE F. Note that these formulas are consistent with the hypothesis of chromosome losses from a tetraploid ancestor with n=12.

Thus the above-discussed species complex exhibits several interesting features:

(i) *Hybridization combined with polyploidy.* The extant species of the group constitute a classic case of alloploidy. Stebbins (1950, 1970) and Grant (1971) have discussed the general importance of this phenomenon in the "reticulate" speciation patterns of higher plants.

(ii) *Known genome composition of the extant alloploid species.* The relationships of *B. carinata, B. juncea* and *B. napus* to their progenitor species have been demonstrated experimentally, not only by genome analysis but also by artificial resynthesis.

(iii) *A second, more ancient level of polyploidy.* The extant "diploids" are apparently auto- or alloploid derivatives of extinct ancestral forms, and have become "secondary balanced" to behave cytologically as diploids.

(iv) *Aneuploidy.* The integral series of gametic chromosome numbers in *B. nigra, B. oleracea* and *B. campestris* is perhaps the result of differential chromosome loss from a postulated ancestor with n=12.

A powerful tool for the study of genetic processes in evolution is the so-called "zymogram" technique. This analytical procedure was pioneered by Hunter and Markert (1957), and is carried out by first separating the components of a protein mixture in a gel matrix under the influence of an electric field. The various protein species migrate at different rates based on their net molecular charge and sometimes their steric interaction with the gel support. In this way, slight structural differences between proteins are translated into migration to characteristic relative positions in the gel under a defined set of conditions of time of migration, temperature, pH, etc. The separation is then followed by the application of a chemical staining mixture designed to visualize zones of catalytic activity for particular enzymes of interest. The result is a pattern of coloured bands on the gel, representing some components of the structural heterogeneity between enzyme molecules with similar catalytic function.

The main advantages of the technique for genetic approaches to evolution consist in:

(i) The reliable way in which homologous genetic information is indicated by structural and functional similarity of phenotype. Stated differently, the presence in divergent organisms of enzymes having similar catalytic and physical (electrophoretic) properties can often be used to infer that the enzymes' structures are specified by DNA sequences with a common ancestry. This inference of genetic homology is more reliable the more closely related are the organisms, and the more rigorously defined are the catalytic and functional properties being used for identification of the enzymes.

This is perhaps the most fundamental advantage of the technique, since it dramatically simplifies the task of tracing, between divergent biological groups like species, the evolutionary fates of information carried by specific gene loci (Lewontin 1974). When using information from higher levels of phenotypic expression such as physiology or morphology, effects of individual homologous loci tend to be obscured by gene interactions like dominance and epistasis. Environmental modification of phenotype presumably plays a greater role at this level as well.

(ii) The frequent possibility of deriving preliminary information on the genetics of enzyme proteins (e.g. number of structural gene loci, presence or absence of allelic variants, levels of heterozygosity), simply by inspection of banding patterns on stained gels. Gottlieb (1977a) gives a clear discussion of the ways in which banding patterns reflect various systems of genetic control of enzymes. Where feasible, such preliminary conclusions may be confirmed by inheritance tests.

(iii) The relative ease and speed with which specific enzymic components of even very complex mixtures can be studied. Crude homogenates are usually adequate for the purposes stated in (i) and (ii).

These three features of the zymogram technique greatly enhance the precision with which many genetic aspects of evolution can be studied, and render practicable surveys of groups the size of populations and species.

In Lewontin (1974), Markert (1975) and Ayala (1976) several of the major applications of the technique to evolutionary questions are reviewed. These include: measurements of levels of genic variation in natural populations (Levin 1975; Lewontin and Hubby 1966), quantitative analyses of geographic variation and population structure (Clegg and Allard 1972; Eanes and Koehn 1978; Hamrick and Allard 1972; Schaal and Levin 1977), and studies of genetic differentiation during speciation (Ayala 1975; Gottlieb 1976).

This approach also holds potential for an improved understanding of the genetics of plant polyploids in relation to the genomic contributions of their diploid progenitors. However, in spite of the long-standing recognition of this potential (Hall 1959; Hall and Johnson 1963), such work has not been plentiful.

Many electrophoretic studies of polyploids have been chemotaxonomic in intent, and have not involved any attempt at genetic interpretation of banding patterns (Reddy and Garber 1971; Sheen 1972; Smith *et al.* 1970; Vaughan *et al.* 1970; Vaughan and Waite 1968). Other workers have asked general genetic or quasi-genetic questions without being concerned with contributions of specific gene loci to banding patterns of the polyploids (Barber 1970; Barber *et al.* 1968; Hall 1959; Houts and Hillebrand 1978; Mitra and Bhatia 1970). Of major concern here have been: increased zymogram complexity with higher ploidy, display of "additive" versus "novel" patterns in polyploids with respect to progenitors, and presence or absence of "hybrid bands" due to cross-reaction of divergent polypeptide subunits coded by loci of component genomes.

Genetic investigation of polyploids using electrophoresis has been carried furthest in material where phenotype is analyzable in terms of expression of individual gene loci. If these loci can then be identified as belonging to particular progenitor genomes, very precise questions can be asked concerning the evolutionary fates of genes in the polyploids.

It is here that zymogramming has proven to be a most incisive approach. Although electrophoretic study of non-enzymic components such as seed storage proteins can reveal gene markers (Garcia-Olmedo *et al.*

1976), in most cases enzymes are more useful for this purpose since homologies and structural locus control are more easily pinpointed.

The organism most thoroughly analyzed in this way to date is common wheat, *Triticum aestivum*. Zymogram studies of the extensive series of aneuploid lines developed by Sears (1954, 1966) have greatly clarified questions surrounding the evolutionary fates of component ("homoeologous") genomes in this allohexaploid. Brewer *et al.* (1969) and Barber *et al.* (1968) were first to apply Sears' nullisomic-tetrasomic "compensation lines" to locate enzyme structural loci on specific homoeologues of a chromosome group in *T. aestivum*. Since then, a large number of such loci have been analyzed by this method (Garcia-Olmedo *et al.* 1978; Hart 1970, 1973, 1975; Hart and Langston 1977a; Torres and Hart 1977; Wolf *et al.* 1977).

Several interesting results have accrued. Hart and co-workers studied a sample of enzyme structural genes belonging to 10 isoenzyme systems in wheat and traced them to specific chromosomes of the *A*, *B* and *D* genomes (Hart and Langston 1977a; Torres and Hart 1977). In all of these 10 systems, structural gene expression was evidently retained in all three genomes of the hexaploid. It should be mentioned, however, that other investigators have detected banding patterns in the same material which suggest non-expression or quantitatively unequal expression of some homoeologous seed protein loci (Garcia-Olmedo *et al.* 1978).

Another interesting fact is that in many of these enzyme systems, "hybrid isozymes" were present, indicating that polypeptide subunits coded by divergent homoeologous structural loci were still similar enough to combine and form a catalytically active multimer.

Another aspect of gene evolution in polyploids which has proven acessible by these techniques is the evolution of interaction between nonallelic genes. Wolf *et al.* (1977) found that the structural genes for phosphodiesterase were located on group 3 chromosomes in wheat, but that a gene or genes on chromosome 5B was necessary for the expression of the structural gene on chromosome 3D. The exciting possibility is that this type of finding may yield insights into the evolution of gene regulation in polyploids.

Roose and Gottlieb (1976) have carried out an interesting analysis of a polyploid complex in *Tragopogon*. Three diploid species have given rise in the present century to two allotetraploid species. Not only are the genome compositions of the alloploids conclusively known, but their recent origin permits an unusually rigorous comparison with the diploid progenitors. These five species were examined electrophoretically for 13 enzyme systems, and gene loci of the tetraploids identified as originating from one or the other progenitor. A large proportion of the duplicate loci in the tetraploids were found to be fixed in a permanently heterozygous condition for electrophoretically detectable alleles, and some enzyme systems displayed extensive hybrid multimer formation.

Other smaller studies have investigated one or a few enzyme systems in alloploids in this way (Cherry *et al.* 1972; Efron *et al.* 1973; Gottlieb 1973). But in general, the zymogram technique remains to be fully exploited in finding answers to such questions as the following:

(i) To what extent are gene products of component genomes expressed in alloploids? One can expect as a working hypothesis that enzymes of both parents tend to be expressed.

(ii) How important are nonparental components of enzyme phenotype which may emerge in polyploids as a consequence of their multiplied chromosome number and/or hybrid origin? Into this category fall such phenomena as nonparental hybrid multimers and altered tissue expression of parental loci.

(iii) How do gene expression patterns evolve subsequent to the origin of a polyploid lineage? Some have proposed (e.g. Ferris and Whitt 1977a, b) that expression of redundant gene loci in polyploids will gradually be lost.

Other problems could be listed and speculations voiced as to the implications of their possible solutions. Suffice it to say here that there is an undeniable need of basic comparative zymogram data on polyploids and their progenitor species. The author's opinion is that the most significant applications of the technique to problems of polyploid evolution will appear in a genetic context.

The present investigation is a comparison of various *Brassica* types belonging to the alloploid complex described above. The objective was to trace the expression of enzyme loci in the various species, using the zymogram technique. Previous electrophoretic studies in *Brassica* have largely emphasized chemotaxonomic questions (Denford and Vaughan 1977; Vaughan and Denford 1968). Comparisons between the allotetraploids and their progenitor species with respect to band similarity and pattern complexity have been carried out (Vaughan *et al.* 1970; Vaughan and Waite 1967), but genetic questions were not asked. Nor is it obvious how the techniques used in these comparisons could be adapted to genetic questions. In all cases, bulk samples of 100-200 dry seeds were used to secure a standard physiological state. But in obligately cross-fertilizing species like the diploid *Brassicas*, considerable between-individual variation in banding phenotype is to be expected for enzymes displaying allelic variation. Thus allelic and nonallelic isozymes tend to be confused in zymograms resulting from such samples, and genetic data (especially in polyploids) would be difficult to obtain. Furthermore, relatively harsh and lengthy procedures are necessary to extract soluble proteins efficiently from dry seeds. This would seem to heighten the possibility of enzymatic and nonenzymatic degradation of sample components, leading perhaps to generation of some new bands or loss or weakening of activity in others. This might even lead to spurious differences between taxa.

With these considerations in mind, the author decided to work with tissues of individual growing plants, in order to clarify the nature of zymogram phenotypes in *Brassica*. Also, to enhance isozyme resolution, a rather unconventional technique of vertical gel electrophoresis was used. Thus, in addition to its being the first known attempt at genetically-framed zymogram analysis in *Brassica*, the present study incorporates certain technical refinements of electrophoresis. These refinements will be discussed more fully under "Materials and Methods" below.

The zymogram phenotype data obtained were used to arrive at workable hypotheses of enzyme locus expression in species of the alloploid complex, in order to address the following two problem areas. First, an attempt was made to determine whether zymogram data could be used to study the structural relationships of the *a* and *c* genomes. Of interest here was possible evidence for ancient polyploidy and/or differential duplication of chromosomes in the two genomes (see formulas on page 4). Secondly, the author sought information on isozyme profile evolution in *B. napus* and its genetic basis (see questions (i), (ii), and (iii) on page 11).

For these purposes, standard diploid cultivars of *B. oleracea* and *B. campestris* (sensu lato: see Olsson 1954 for the taxonomic treatment of 10-chromosome forms which is used herein) were analyzed, as well as a standard tetraploid cultivar of *B. napus*. In addition to these, synthetic autotetraploids of *B. oleracea* and *B. campestris*, and synthetic *B. napus* derived from a cross between the two autotetraploid stocks, were examined.

MATERIALS AND METHODS

Sources of Plant Material

All plants in this study were grown from seed. The varieties used are listed in Table 2, along with their sources. Seeds of *Brassica campestris* ssp. *pekinensis* were obtained from the same source as those of diploid *B. campestris* ssp. *chinensis*. Identities of plants were confirmed by examination of resette leaf characters (Sun 1946a, b). No difficulty was encountered in distinguishing on this basis between the various taxa. Seed voucher specimens are deposited with Dr. K. E. Denford, Department of Botany, University of Alberta.

Seedling Germination and Growth

Thirty to one hundred dry seeds of a given variety were allowed to imbibe water for 4-5 hours, then germinated on filter paper in sterile plastic Petri dishes in a controlled environment chamber. No fungicidal or bactericidal treatments appeared necessary. Fluorescent lighting (Westinghouse Cool White) was employed; photoperiod during this stage was 16 hr. (25°C) and dark period 8 hr. (10°C). See Appendix 2 for the spectrum of photosynthetically active radiation obtained from these lamps. Relative humidity was maintained at 70%. Germination under these conditions exceed 80% for all varieties, and often approached 100%.

Seedlings 5-10 days old were transplanted (4 to a pot) to 6" plastic pots filled with a 1:1:1 mixture of sand, soil and sphagnum moss. Growth from this point onward took place in a walk-in growth chamber, with 16 hr. photoperiod (20°C) and 8 hr. dark period (10°C). Fluorescent lighting (Sylvania Cool White) was employed during this stage also and relative humidity was maintained at ca. 50%. Plants were kept well watered.

Abbreviations Used in the Text for Chemical Names

Abbreviation	Full Name
DDW	Water, twice-distilled in glass and deionized
Disodium EDTA	Disodium salt of ethylene diamine tetraacetic acid
DTT	Dithiothreitol
MTT	Thiazolyl Blue
NAD^+	β-nicotinamide adenine dinucleotide
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NBT	Nitro blue tetrazolium
PMS	Phenazine methosulfate
PVP-10	Soluble polyvinylpyrrolidone, avge. mol. wt. 10,000
Tris	Tris-(hydroxymethyl) aminomethane

Table 2

Names, genome formulas and sources of taxa of *Brassica* compared.

GROUP I

Taxon	Genome Formula	Source
Brassica campestris L. ssp. *oleifera* (Metzg.) Sinsk. *f. biennis* 'Rapido II'	*aa*	K. Downey, Agriculture Canada Research Station, Saskatoon, Saskatchewan, Canada
Brassica oleracea L. var. *acephala* DC. 'Borecole Dwarf Green Curled'	*cc*	Robertson Seeds Ltd., Edmonton, Alberta, Canada
Brassica napus L. ssp. *oleifera* (Metzg.) Sinsk. *f. biennis* 'Argus'	*aacc*	W. A. Keller, Agriculture Canada Research Station, Ottawa, Ontario, Canada

GROUP II

Taxon	Genome Formula	Source
Brassica campestris L. ssp. *chinensis* (L.) Makino (diploid)	*aa*	Chr. Lehmann, Zentralinstitut für Genetik und Kulturpflanzenforschung der Akademie der Wissenschaften der DDR, Gatersleben, Germany
Brassica campestris L. ssp. *chinensis* (L.) Makino (autotetraploid)	*aaaa*	G. Olsson, Swedish Seed Association, Svälof, Sweden
Brassica oleracea L. var. *acephala* DC. 'Marrowstem Kale' (diploid)	*cc*	B. R. Stefansson, University of Manitoba, Winnipeg, Manitoba, Canada
Brassica oleracea L. var. *acephala* DC. 'Marrowstem Kale' (autotetraploid)	*cccc*	G. Olsson (see above)
Synthetic *Brassica napus* L. 2236 (from ssp. *chinensis* 4N x Marrowstem Kale 4N	*aacc*	G. Olsson (see above)

Leaf Extracts

Material for electrophoretic analysis was taken from one or the other of the first two true leaves to emerge. Only fully expanded leaves showing no visible signs of senescence were included. Samples from individual plants were treated separately throughout the analyses.

For analysis on polyacrylamide gels, 50-200 mg fresh weight of tissue per leaf was enclosed in a tared vial and weighed. This tissue was then homogenized in a closely fitting ground glass homogenizer in 5 ml of ice cold extraction buffer per gram fresh weight of tissue. The extraction buffer was 0.06 M Tris - HCl (molarity here refers to chloride), pH 6.7, and contained: PVP-10 (8% w/v), sucrose (10% w/v) disodium EDTA (0.17% w/v), DTT (0.015% w/v), NAD^+ (0.005%), $NADP^+$ (0.005% w/v), and pyridoxal-5'-phosphate (0.005% w/v). The crude homogenates were then centrifuged at 17,000 X g for 40 minutes at 4°C to clear them of cellular debris. The supernatant fraction after this treatment was a pale yellow colour, and clear (i.e. it had the appearance of the extraction buffer itself). Thus, no appreciable quantities of pigments apparently were released on grinding. No lipid flocculent appeared on the surface of the extract. These supernatants were removed with pipettes, transferred to small vials and stored on ice in the dark for up to 2 hours until subjected to electrophoresis.

Electrophoresis

All enzymes were analyzed in polyacrylamide gels, using a multiphasic gel and buffer system closely resembling that described by Davis (1964). The only differences were as follows:

(i) The spacer gel polymerization mixture was 5.0% (final concentration) with respect to acrylamide and 2.5% (final concentration) with respect to bisacrylamide. The final concentration of riboflavin in the polymerization mixture was correspondingly increased to 1.25%. These concentrations were achieved for the acrylamide and bisacrylamide simply by using a twofold instead of a fourfold dilution of the usual 10%/2.5% stock solution, and for the riboflavin by performing eightfold dilution of a 10% stock solution, in contrast to the usual 5% stock solution. This resulted in a spacer gel with smaller "pore size" but with improved mechanical strength, which was essential for formation of sample slots in the slab-format gels.

(ii) The samples were layered in solution directly on the spacer gel surface, instead of being polymerized into a sample gel.

Disc electrophoresis was carried out in glass tubes of 5 mm. internal diameter in a Buchler "Polyanalyst" apparatus, in which ice water was circulated to maintain a buffer temperature below 7°C throughout the run. Running gels were approximately 50 mm long, and spacer gels 10 mm long. Thirty to seventy microliters of sample was layered under pre-chilled cathode buffer onto the top of the spacer gel, using a 100-microliter syringe. Constant current of 1.5 mA per tube was applied until the marker dye was seen to have entered the spacer gel; the current was then raised to 2.5 mA per tube and maintained at that level until the marker dye had migrated the desired distance from the origin. At this point, the current was discontinued, and the gels extruded into staining tubes containing DDW at room temperature. When all gels had been removed thus from their electrophoresis tubes, the DDW was decanted from the staining tubes and stain added to all gels simultaneously.

Slab-format gels using the same gel and buffer system as that employed for disc electrophoresis were run in an apparatus constructed as illustrated in Appendix 1. The running gels for this format were approximately 90 mm long, with spacer gels about 15 mm long and sample wells approximately 20 mm deep. Thirty to fifty microliters of sample was layered under cathode buffer into the sample wells, which were cast in the spacer gel. Constant current of 15 mA per slab was applied until all samples had entered the spacer gel; the current was then raised to 30 mA per slab and maintained at that level until the dye front had migrated the desired distance. Temperature was maintained below 10°C throughout the run by pre-chilling the anode buffer surrounding the gels to 3°C, and conducting the entire separation in a refrigerator. When electrophoresis was completed, the slab gels were freed from the constraining glass plates and stained with the same reagents as were the disc gels.

Enzyme Stains

The following recipes are slightly modified versions of those given by Brewbaker *et al.* (1968), Shaw and Prasad (1970), and Siciliano and Shaw (1976) for enzymes separated in starch gels. Where appropriate, mention is made of points of technique which seemed essential for efficient staining with this plant material and electrophoresis system.

Buffers used in staining mixtures were of three types:

(A) 0.2 M Tris-HCl, pH 8.2

1 M Tris .... 200 ml

adjust to pH 8.2 with 1N HCl

make up volume to 1 liter with DDW

(B) 0.5 M Phosphate, pH 7.0

1 M K_2HPO_4 .... 500 ml

adjust to pH 7.0 with 1 M KH_2PO_4

make up volume to 1 liter with DDW

(C) 0.5 M Acetate, pH 5.0

1 M Na Acetate .... 500 ml

adjust to pH 5.0 with glacial acetic acid

make up volume to 1 liter with DDW

These are referred to in the recipes below as stain buffers "A", "B", and "C". Unless otherwise specified, recipes are for 100 ml of stain, and sample volumes apply to *Brassica* leaf tissue homogenized in the 5:1 ratio of buffer volume to tissue weight mentioned above.

(1) Alcohol Dehydrogenase (ADH) - E.C. 1.1.1.1

Stain Buffer A	20 ml
95% ethanol	5 ml
DDW	75 ml
NAD^+	60 mg
NBT	40 mg
PMS	2 mg

Stain overnight in dark at 37°C.

ADH activity in rosette leaves of *Brassica* apparently was not high in the conditions under which the plants were grown. Seventy to one hundred microliters of sample were applied to disc gels to obtain satisfactory staining reactions.

(2) Catalase (Cat) - E.C. 1.11.1.6

Solution 1:	0.5% H_2O_2	100 ml
Solution 2:	1% KI, acidified with 0.5 ml of glacial acetic acid	100 ml

Apply solution 1 to gel for 1 minute. Pour this off and apply solution 2. The bands appear as achromatic zones in a blue background after a few minutes at room temperature. To apply this stain to catalases separated in polyacrylamide, the gel is cast containing 0.5% soluble starch. Sample volume 50 microliters.

(3) Esterase (Est) - E.C. 3.1.1.1

Stain:	Stain buffer B	50 ml
	Esterase substrate*	3 ml
	DDW	47 ml
	Fast Blue RR Salt	100 mg

*Esterase Substrate (1% α - naphthyl acetate):

α - naphthyl acetate ..... 1 g

dissolve in 50 ml acetone; make up volume to 100 ml with DDW.

Stain in dark at room temperature. Sample volume 50 microliters.

(4) Glucose-6-Phosphate Dehydrogenase (G6PD) - E.C. 1.1.1.49

Stain Buffer B	20 ml
DDW	79 ml
10% $MgCl_2$	1 ml
D-Glucose-6-phosphate (monosodium salt)	300 mg
$NADP^+$	30 mg
NBT	30 mg
PMS	2 mg

Incubate at 37°C in the dark. Activity tends to be rather low in this material. Sample volume 100 microliters.

(5) Glutamate Dehydrogenase (GDH) - E.C. 1.4.1.4

Stain:

Stain Buffer B	25 ml
GDH Substrate*	25 ml
DDW	50 ml
NAD^+	100 mg
NBT	35 mg
PMS	2 mg

*GDH Substrate (1M Na-L-Glutamate, pH 7.0):

L-glutamic acid (monosodium salt) .... 16.9 g

dissolve in 60 ml of Stain Buffer B

make up volume to 100 ml with Stain Buffer B.

Incubate overnight at 37°C. Sample size 50 microliters.

(6) Glutamate-Oxaloacetate Transaminase (=Aspartate Aminotransferase) (GOT) - E.C. 2.6.1.1

Stain:	GOT Substrate*	100 ml
	pyridoxal-5'-phosphate	5 mg
	Fast Blue BB Salt	200 mg

*GOT Substrate:

L-aspartic acid	5.30 g
α-ketoglutaric acid	700 mg

dissolve in 500 ml of Stain Buffer A

make up to 1 liter with Stain Buffer A.

The GOT Substrate solution is stable for several months if refrigerated in a brown bottle.

Fast Blue BB Salt begins to degrade immediately upon its addition to this staining mixture, and is essentially inactive after 15 minutes. For this reason, the salt should be added last to the staining mixture with vigorous stirring, and the gel should be ready for rapid application of the stain as soon as the salt has been dissolved. The gel is covered, placed in the dark and allowed to develop at room temperature. Sample size 30 microliters.

(7) Indoleacetic Acid Oxidase (IAO)

Stain Buffer C	95 ml
95% ethanol	5 ml
3% H_2O_2	0.1 ml
indole-3-acetic acid	17.6 mg
p-coumaric acid	16.4 mg
Fast Blue BB Salt	100 mg

Dissolve indole-3-acetic acid and p-coumaric acid in 95% ethanol. Add buffer and H_2O_2. Dissolve Fast Blue BB and apply stain to gel; incubate in the dark at room temperature. Fast Blue BB Salt is quite stable in this staining mixture. Zones of IAO activity appear as brownish-to-whitish bands. An extraction buffer: tissue ratio of 1 ml:1 g was used for IAO (see recipe for Peroxidase below). Sample volume 50 microliters.

(8) Isocitrate Dehydrogenase (IDH) - E.C. 1.1.1.42

Stain Buffer A	20 ml
DDW	79 ml
10% $MgCl_2$	1 ml
$NADP^+$.........................	30 mg
NBT	30 mg
PMS	2 mg
DL-isocitric acid (trisodium salt)	50 mg

Apply stain to gel; cover and allow to stand at room temperature for 5 minutes. Then incubate at 37°C in the dark until blue bands appear. Sample size 30 microliters. NOTE: Mn++ was not effective as a cofactor for soluble IDH in *Brassica*, under these staining conditions

(9) Leucine Aminopeptidase (LAP) - E.C. 3.4.3.1

For 200 ml of stain:

Stain Buffer B	40 ml
DDW	150 ml
Dimethyl formamide	10 ml
L-leucyl-β-naphthylamide	20 mg
Fast Garnet GBC Salt	30 mg

Dissolve L-leucyl-β-naphthylamide in dimethyl formamide. Dissolve Fast Garnet in DDW and buffer. Add salt solution to naphthylamide solution; apply to gel. Cover and allow to stain overnight at room temperature. Buffer:tissue ratio 1:1; sample volumes 50 microliters.

(10) Malate Dehydrogenase (MDH) - E.C. 1.1.1.37

Stain:	Stain Buffer A	20 ml
	DDW	70 ml
	MDH Substrate*	10 ml
	NAD^+	66 mg
	NBT	35 mg
	PMS	2 mg

*MDH Substrate (1 M Na-L-malate, pH 7.0):

L-malic acid	13.4 g

dissolve in DDW

adjust pH to 7.0 with 2M $Na_2CO_3 \cdot H_2O$ while swirling in an ice bath

Incubate at 37°C in the dark. Care is taken not to overstain, as MDH activity in this material is quite high. Sample volume 30 microliters.

(11) Peroxidase (Per) - E.C. 1.11.1.7

Stain Buffer C	92.5 ml
3% H_2O_2	2.5 ml
Dimethyl formamide	5 ml
3-amino-9-ethyl carbazole	50 mg

Dissolve 3-amino-9-ethyl carbazole in dimethyl formamide. Add H_2O_2 to buffer. With gel ready, slowly add buffer solution to dimethyl formamide solution, with stirring. Apply immediately to gel, cover, and allow to develop at room temperature. An extraction buffer: tissue ratio of 1 ml:1 g was used for extraction in Peroxidase. Sample volume 30-50 microliters.

(12) Phosphoglucomutase (PGM) - E.C. 2.7.5.1

Stain Buffer A	20 ml
DDW	79 ml
10% $MgCl_2$	1 ml
α-D-glucose-1-phosphate (disodium salt)	600 mg
$NADP^+$.........................	30 mg
MTT	40 mg
PMS	2 mg
Glucose-6-phosphate dehydrogenase	180 units

Incubate at 37°C in the dark. The G6PD should be added at the last second before application of the stain to the gel. Sample size 30 microliters.

(13) Phosphoglucose Isomerase (PGI) - E.C. 5.3.1.9

Stain Buffer A	50 ml
DDW	49 ml
10% $MgCl_2$	1 ml
D-Fructose-6-phosphate (sodium salt)	160 mg
$NADP^+$.........................	20 mg
MTT	20 mg
PMS	2 mg

Glucose-6-phosphate
dehydrogenase .... 180 units

Treat as with PGM. PGI activity is very high in this material, with bands emerging in 3-5 minutes after application of the stain. Care should thus be taken not to overstain.

(14) Superoxide Dismutase (SOD)

When staining for IDH has proceeded to a satisfactory intensity (see stain recipe #8), uncover the gel and place under a fluorescent lamp for 1 hr. Achromatic zones of non-photoreduction of NBT represent SOD activity.

Recording of Results

Isozyme banding patterns were recorded immediately upon completion of staining, in the forms of (a) photographs and (b) interpretive drawings. Most stained gels could be stored for limited periods in distilled water. No success was had in attempting to dry them for permanent records.

For photography, stained disc gels were placed in distilled water in stoppered glass tubes. Slab gels were placed without added water into transparent rectangular polystyrene boxes. The light source was a 40 watt incandescent bulb, shielded by a 1/8" thickness of white acrylic plastic. Photographs were taken with a Pentax KX 35 mm camera, equipped with a 50 mm "macro" lens; Kodak Panatomic X black and white film was used. In some cases it was possible to enhance contrast of isozyme bands with respect to a coloured gel background, by the use of photographic filters of the same colour as the gel background. For example, gels stained for GOT activity with the staining mixture described in the previous section characteristically display blue bands on an orange-to-red background. Use of a red filter sharpened contrast here.

Interpretive drawings were made according to the protocol described in Chapter 3.

RESULTS

All of the 14 enzymes examined in the leaf extracts, except isocitrate dehydrogenase, displayed isozymic forms upon disc electrophoresis. An initial indication of the complexity and between-species variability of each isozyme system was gained from these analyses, for which the taxa of Group I were used. The results are summarized in Table 3. No attempt was made to survey isozyme variation within taxa at this stage; the goals were simply to (i) establish efficient extraction, electrophoresis and staining procedures, and (ii) select enzyme systems suitable for more detailed comparisons using slab-format gels.

Phosphoglucomutase (PGM), superoxide dismutase (SOD), glutamate dehydrogenase (GDH), glutamate-oxaloacetate transaminase (GOT) and isocitrate dehydrogenase (IDH) were selected for further analysis on the basis of the following criteria. These enzymes all displayed conveniently high levels of activity in the disc gel analyses of leaf extracts, and staining reagents were not excessively costly. They were also chosen to represent a variety of catalytic activities, metabolic roles and complexities of molecular structure. Finally, two of the enzymes (SOD and GOT) were included because they displayed between-sample variation in isozyme pattern in disc electrophoresis, and thus promised to yield data on their modes of structural gene control.

Slab-gel comparisons of zymogram profile were made (i) between the members of Group I, and (ii) between the members of Group II. By

Table 3. Enzyme activities detected by disc electrophoresis zymograms in *Brassica* leaf extracts.

Enzyme	E.C.	Taxa Analyzed*	Number of Isozymes
Alcohol Dehydrogenase	1.1.1.1	Bc, Bo, Bn	2
Catalase	1.11.1.6	Bc, Bo, Bn	2
Esterase	3.1.1.1	Bc, Bo, Bn	8-10
Glucose-6-Phosphate Dehydrogenase	1.1.1.49	Bc, Bo, Bn	4
Glutamate Dehydrogenase	1.4.1.4	Bc, Bo, Bn	7
Glutamate-Oxaloacetate Transaminase	2.6.1.1	Bc, Bo, Bn	3-12
Indoleacetic Acid Oxidase	unclassified	Bc, Bo, Bn	2
Isocitrate Dehydrogenase	1.1.1.42	Bc, Bo, Bn	1
Leucine Aminopeptidase	3.4.3.1	Bc, Bo	2
Malate Dehydrogenase	1.1.1.37	Bc, Bo	7
Peroxidase	1.11.1.7	Bc, Bo, Bn	3
Phosphoglucomutase	2.7.5.1	Bc, Bo	3-4
Phosphoglucose Isomerase	5.3.1.9	Bc, Bo	3
Superoxide Dismutase	unclassified	Bc, Bo, Bn	2-4

*Bc = *Brassica campestris*

Bo = *Brassica oleracea*

Bn = *Brassica napus*

parallel electrophoresis in slab gels of samples from different taxa, correspondences could be set up between zymogram phenotypes of any given pair of taxa. After applying this procedure to SOD, PGM and GDH isozymes it proved practicable and informative to assign individuals within taxa to phenotypic classes, and to compare these classes between taxa. These results are summarized illustratively in Figures 2-15. In the diagrams, relative band mobilities are indicated by the lower edges of the symbols. Subjective estimates of relative band intensity within the zymogram profile of an individual are represented by the widths of the bars; ambiguity with regard to presence or absence of a band is symbolized by a dotted line.

Comparisons of zymogram band intensities between samples run in parallel on a gel should be undertaken cautiously, since small imprecisions in extraction and handling procedure may lead to variations from sample to sample with respect to staining behaviour. For this reason, phenotype classification was carried out here on the basis of differences in relative band intensity between samples, and was restricted to cases where discrete variation was seen in the isozyme pattern . These rules of interpretation were followed in constructing Figures 3, 4, 10, 11, 13 and 14, and the validity of this approach is considered further in Chapter 4.

It was not possible to apply this interpretive procedure to the results from GOT or IDH. These results are therefore presented in photographic form, with some salient points of the zymograms being pointed out which may prove useful in future analyses of these systems.

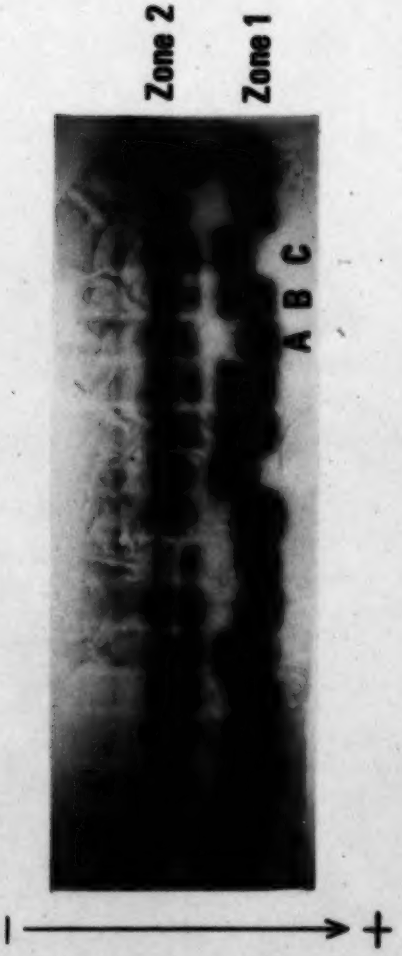
In the following five sections of the text, the data from the five enzyme systems are summarized for Groups I and II. For ease of discussion, the names of the taxa were abbreviated: the members of Group I are referred to as Rapido II, Borecole and Argus, and those of Group II as Chinensis 2N, Chinensis 4N, Marrowstem 2N, Marrowstem 4N, and Synthetic napus. Isozyme phenotypes were given names based on the abbreviated name of the enzyme plus a superscript. The taxon in which the phenotype occurs is specified by a prefixed abbreviation of the taxon name. For example, SOD phenotype A of Chinensis 2N is referred to as Chin 2-SOD^A. The abbreviated prefixes for the other taxa of Group II are "Chin 4", "Marrow 2", Marrow 4", and "Syn". Those for the taxa of Group I are "RII", "Bore", and "Arg".

Isozymes were numbered according to IUPAC-IUB rules of nomenclature (Anonymous, 1972), in order of decreasing anodal mobility. Series of isozymes were numbered independently for each taxon. In cases of suspected overlap of isozyme mobilities, the overlapping components were given separate numbers only if they were observed separately in different samples.

Phosphoglucomutase

PGM activity was distributed between two electrophoretic zones in all taxa examined. This zonation is clearly evident in Figure 2, in which the zymogram phenotypes of 16 co-electrophoresed plants of *B. campestris* ssp. *pekinensis* are shown. Band mobilities varied independently in the two zones, which were named zone 1 and zone 2 in decreasing order of anodal mobility. Unfortunately, the zone 2 isozymes of *B. oleracea* were not consistently stainable (Fig. 6, p. 41),

Figure 2. Photograph of slab gel containing extracts from 16 individuals of *Brassica campestris* ssp. *pekinensis*, stained for phosphoglucomutase activity. Two independently varying zones of PGM activity are clearly visible. A-phenotype A; B-phenotype B; C-phenotype C. Cathode, anode and direction of migration are indicated on left.


Zone 2
Zone 1
A B C
−
+

so only zone 1 phenotypes could meaningfully be compared between taxa. Zone 1 phenotypic variation was readily classifiable, especially in the diploid taxa. This information is summarized for Group I in Figure 3 and for Group II in Figure 4. Although *B. campestris* ssp. *pekinensis* is not strictly a member of either Group I or Group II, the first PGM slab results to be obtained were with this taxon and the data proved very useful in the interpretation of the phenotypes of the other taxa. They are thus included in the diagram for purposes of later discussion.

In the Group II diploids, aside from individuals of phenotype Chin 2-PGM^B, the number of isozymes per individual was 2. In the Chin 2-PGM^B individuals no staining reaction was visible in zone 1, even though zone 2 activity appeared normal (Fig. 5, p. 41). The Marrowstem 2N isozymes had slightly lower mobilities than those of Chinensis 2N, but no variation was detected within Marrowstem 2N.

Addressing the Group II autotetraploids next, Marrowstem 4N also displayed 2 isozymes. These were of slightly lower mobility than those of Marrowstem 2N. As with Marrowstem 2N, no variant phenotypes were seen within Marrowstem 4N. In contrast, Chinensis 4N showed distinct variation between individuals and band number and relative intensity. Either 2 or 4 bands were observed, and the slower pair of bands varied in intensity relative to the fast pair to give Chin 4-PGM^B and Chin 4-PGM^C. Isozymes 1 and 2 in Chinensis 4N had the same mobilities as isozymes 1 and 2 in Chinensis 2N.

The Marrowstem 2N and Marrowstem 4N enzymes were distinct from those of Chinensis 2N and Chinensis 4N; the two species showed almost

Figure 3. Zone 1 PGM phenotypes of the taxa in Group I. Line and bar symbolism for isozyme bands is explained on page . Phenotypes for each taxon are indicated by letters; phenotypic frequency (numerator) in a sample of size n(denominator) indicated at the top of the figure.

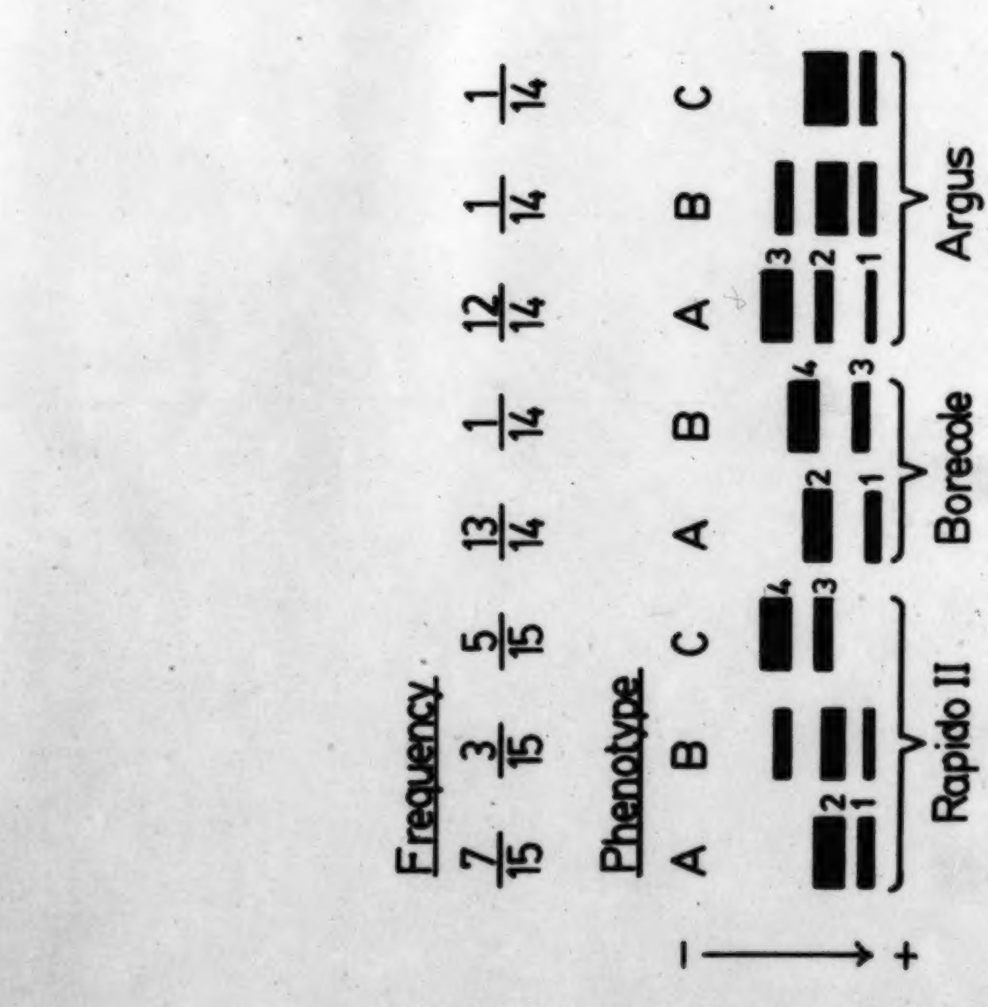

Frequency
7/15
3/15
5/15
13/14
1/14
12/14
1/14
1/14
Phenotype
A
B
C
A
B
A
B
C
−
+
1
2
3
4
1
2
3
4
1
2
3
Rapido II
Borecole
Argus

Figure 4. Zone 1 PGM phenotypes of the taxa in Group II. For explanation of format, see Figure 3.

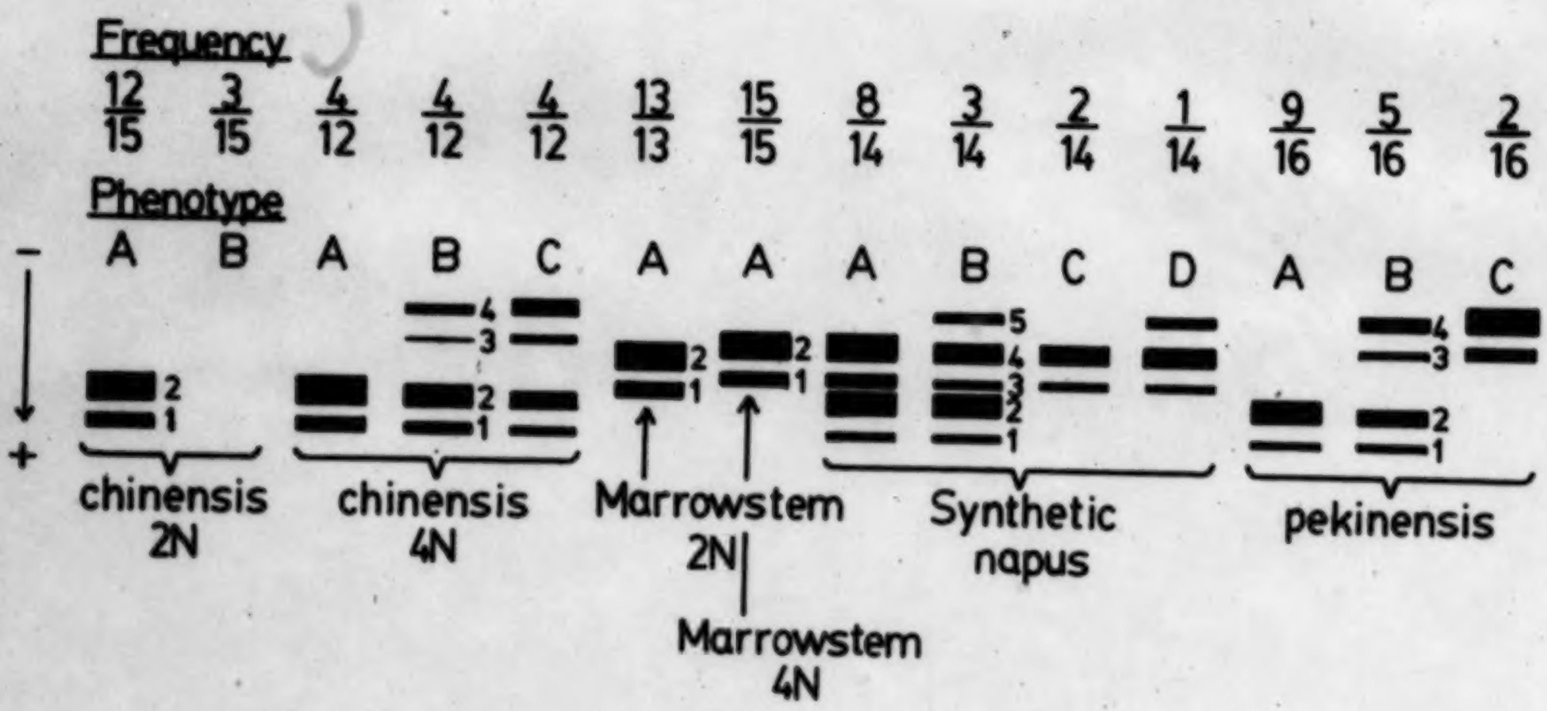
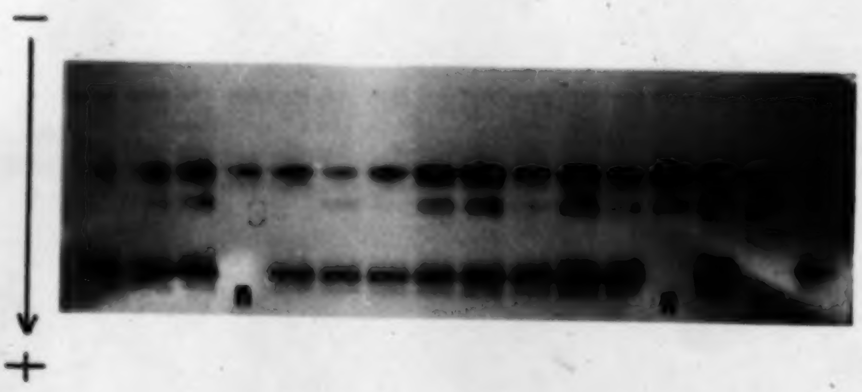
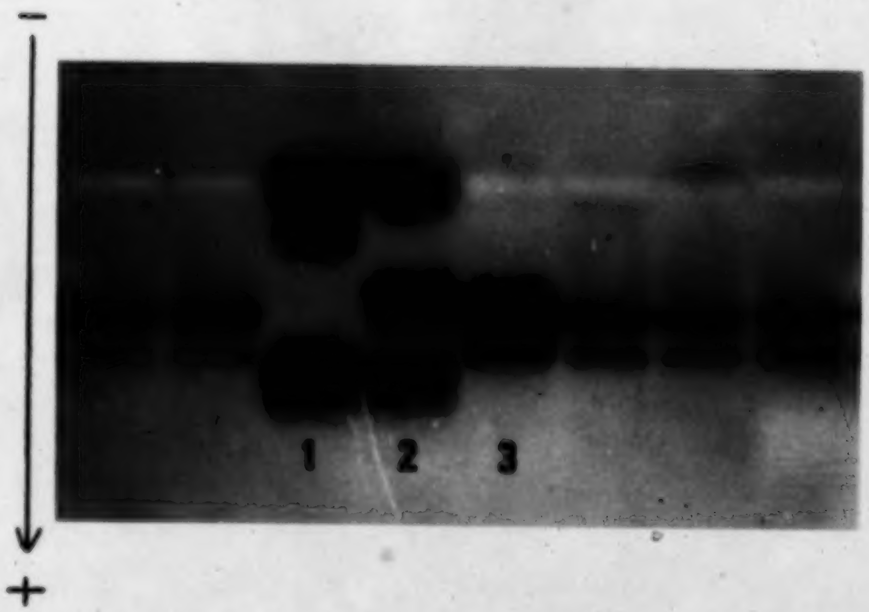

Frequency
12/15
3/15
4/12
4/12
4/12
13/13
15/15
8/14
3/14
2/14
1/14
9/16
5/16
2/16
Phenotype
A
B
A
B
C
A
A
A
B
C
D
A
B
C
−
+
1
2
3
4
5
chinensis 2N
chinensis 4N
Marrowstem 2N
Marrowstem 4N
Synthetic napus
pekinensis

Figure 5. Photograph of PGM gel of diploid *Brassica* ssp. *chinensis*. Symbols 'n' denote individuals with no visible zone 1 activity.

Figure 6. Closeup photograph of phosphoglucomutase gel in which the four diploid and autotetraploid taxa of Group II were co-electrophoresed. 1-diploid *B. campestris* ssp. *chinensis*, phenotype A; 2-autotetraploid *B. campestris* ssp. *chinensis*, phenotype C; 3-autotetraploid *B. oleracea* var. *acephala* 'Marrowstem kale', phenotype A. Remainder of samples are diploid Marrowstem kale, phenotype A. Note: (a) almost complete lack of zone 2 activity in *B. oleracea*; (b) band overlap between samples 2 and 3; (c) slightly slower zone 1 bands in autotetraploid as compared to diploid Marrowstem kale.


−
+


−
1
2
3
+

completely non-overlapping mobilities. The exceptions to this were the overlapping mobilities of isozyme 3 of Chinensis 4N and isozyme 2 of Marrowstem 4N. The relationships between zone 1 PGM isozymes of the diploids and autotetraploids of Group II are displayed in Figure 6, which shows a gel in which all four of these taxa were co-electrophoresed.

Synthetic *B. napus*, with four phenotypes, exhibited the greatest internal variation of the Group II taxa. It also showed the greatest isozymic complexity within individuals, Syn-PGM^B having 5 bands. Novel bands absent in the parental autotetraploid stocks were not seen in Synthetic napus. Since the enzymes of the parental stocks have non-overlapping mobilities, it was possible to identify parental affinities of isozymes in the alloploid. In all phenotypes except Syn-PGM^C, contributions from both parents may be seen clearly. In Syn-PGM^C, only the Marrowstem 4N isozymes are visible. A photograph of a portion of a gel in which Synthetic napus and its two parents were co-electrophoresed is shown in Figure 7.

Turning to Group I, a similar picture emerges. In this case both diploids show internal variation - Borecole with two zone 1 phenotypes and Rapido II with three. Both taxa show a minimum of 2 isozymes. A band of mobility differing from isozymes 1 and 2 of RII-PGM^A was seen in both RII-PGM^B and RII-PGM^C. In addition, varying relative intensity of PGM activity in the second-fastest band was seen in the three Rapido II phenotypes. Isozyme 2 of RII-PGM^A and isozyme 3 of RII-PGM^C were named separately on the basis of their probably distinct biochemical and genetic origins, notwithstanding their close similarity in mobility. This shall be clarified in Chapter 4.

Figure 7. Closeup photograph of phosphoglucomutase gel in which Synthetic *Brassica napus* and parents were co-electrophoresed. 1-autotetraploid *B. campestris* ssp. *chinensis*, phenotype B; 2-autotetraploid Marrowstem kale, phenotype A; A-Synthetic napus, phenotype A; B-Synthetic napus, phenotype B; D-Synthetic napus, phenotype D.

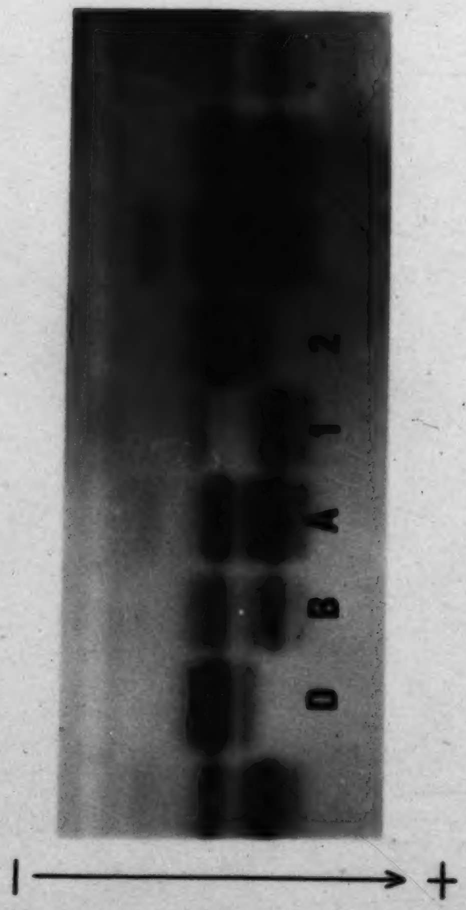
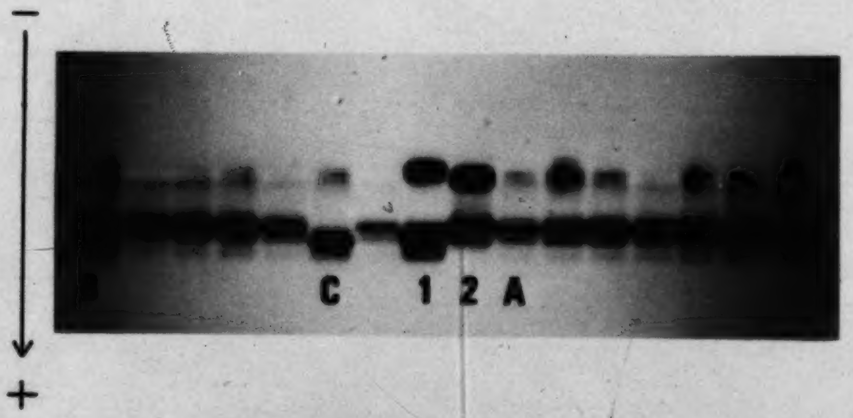
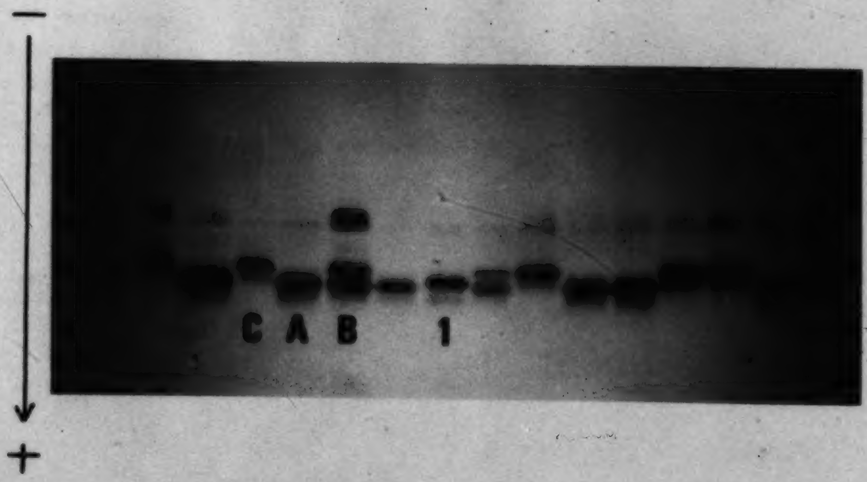

D
B
A
1
2
−
+

Figure 8. Photograph of phosphoglucomutase gel in which the three taxa of Group I were co-electrophoresed. 1-*B. oleracea* var. *acephala* 'Borecole', phenotype A; 2-*B. campestris* ssp. *oleifera* 'Rapido II', phenotype C; A, B, C-*B. napus* ssp. *oleifera* 'Argus', phenotypes A, B, C.

Figure 9. Photograph of phosphoglucomutase gel in which the two diploid taxa of Group I were co-electrophoresed. 1-*B. oleracea* var. *acephala* 'Borecole', phenotype A; A, B, C-*B. campestris* ssp. *oleifera* 'Rapido II', phenotypes A, B, C.


−
C
1
2
A
+


−
C
A
B
1
+

Superoxide Dismutase

Achromatic zones appeared on several types of zymograms in which tetrazolium salts were used as chromogens (dehydrogenases, PGI and PGM). One very slow-migrating zone (zone 2) appeared clearly on GDH gels after overnight staining (see Fig. 15). In addition to this zone, a faster-migrating form was present which appeared quickly during the staining of IDH gels. Since this faster zone (zone 1) corresponded closely in disc-electrophoretic mobility with that of purified copper-zinc SOD from other plant sources (Asada *et al.* 1973; Beauchamp and Fridovich 1973), it was considered most suitable for study in this material. This form showed considerable phenotypic variation within and between taxa, and the results pertaining to this variation are presented in Figures 10 and 11.

In Group II, bands 1 and 2 were consistently present in all individuals of each taxon; no variations between taxa in the mobilities of these bands were detected. Among the diploids, Chinensis 2N was phenotypically invariant, but certain individuals of Marrowstem 2N showed pairs of variant bands which were both faster and slower than bands 1 and 2. The slower bands (3 and 4) were clearly either present or absent in individuals of this taxon, and two phenotypic classes - Marrow 2-SOD^A and Marrow 2-SOD^B were distinguished on this basis. However, no such classes could be clearly demarcated for the faster-migrating pair of bands. A continuous range of staining "intensities" of these bands seemed to characterize the zymograms obtained from plants in this group. They were thus excluded from consideration in constructing the phenotypic classification.

With respect to the autotetraploids, Chinensis 4N was invariant, as was its diploid counterpart. Marrowstem 4N showed one variant phenotype in addition to the more common Marrow 4-SOD^{A}. This phenotype, Marrow 4-SOD^{B}, was characterized by the presence of two variant bands of mobilities apparently identical to those of bands 3 and 4 of Marrow 2-SOD^{B}. These bands were of widths and intensities such that Marrow 4-SOD^{B} had a markedly asymmetrical appearance, as shown in the diagram.

Synthetic napus displayed three phenotypes - the standard two-banded pattern (Syn-SOD^{A}) and two asymmetric types (Syn-SOD^{B}, Syn-SOD^{C}). In Syn-SOD^{B}, two variant bands are clearly present which correspond in mobility to bands 3 and 4 of Marrow 4-SOD^{B}. However, in Syn-SOD^{C} band 3 is weaker than in Syn-SOD^{B}, and band 4 is very weak indeed.

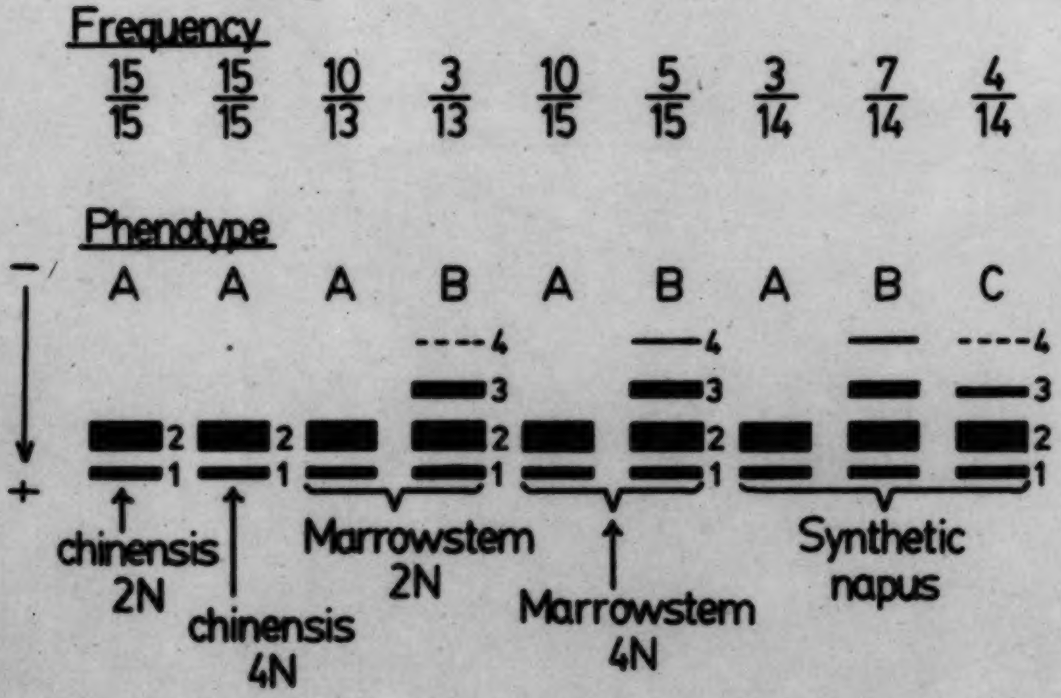
Turning to Group I, the patterns of phenotypic variation are somewhat similar to those of Group II. Both diploids exhibited bands with higher mobilities than bands 1 and 2. As with Marrowstem 2N, these were of continuously variable intensity relative to bands 1 and 2, and thus were not included in the phenotype classification. When these bands are ignored Rapido II is internally invariant. Borecole individuals were mostly Bore-SOD^{A}, but two plants were of variant type Bore-SOD^{B}. In this pattern two slower-migrating variant bands were seen, and the three slowest (2,3, and 4) were of approximately equal intensity.

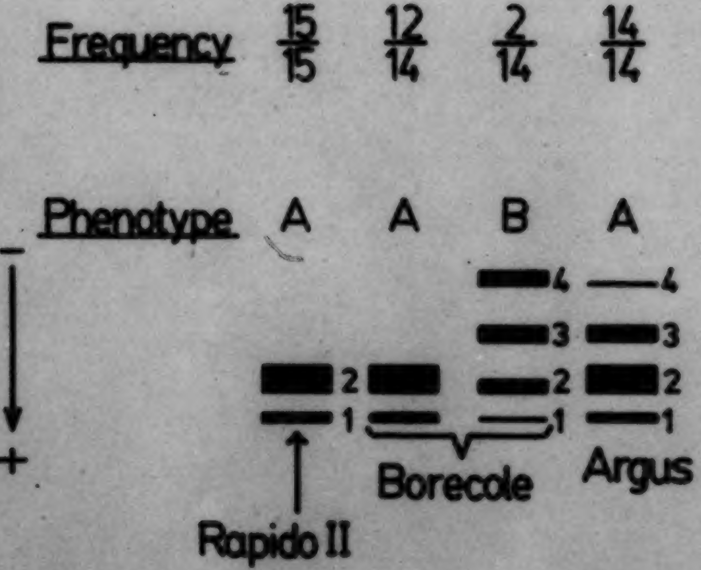
Argus, the allotetraploid representative of Group I, showed a single phenotype in which two slower bands of the same mobilities as bands 3 and 4 of Bore-SOD^{B} were present. Bands 3 and 4 of Arg-SOD^{A} were clearly fainter than band 1, giving this phenotype an asymmetric appearance also.

↑
2

Figure 10. Superoxide dismutase phenotypes of the taxa in Group II. For explanation of format, see Figure 3, p. 37.

Figure 11. Superoxide dismutase phenotypes of the taxa in Group I. For explanation of format, see Figure 3, p. 37.


Frequency
15/15
15/15
10/13
3/13
10/15
5/15
3/14
7/14
4/14
Phenotype
A A A B A B A B C
−
+
4
3
2
1
chinensis 2N
chinensis 4N
Marrowstem 2N
Marrowstem 4N
Synthetic napus


Frequency
15/15
12/14
2/14
14/14
Phenotype
A A B A
−
+
4
3
2
1
Rapido II
Borecole
Argus

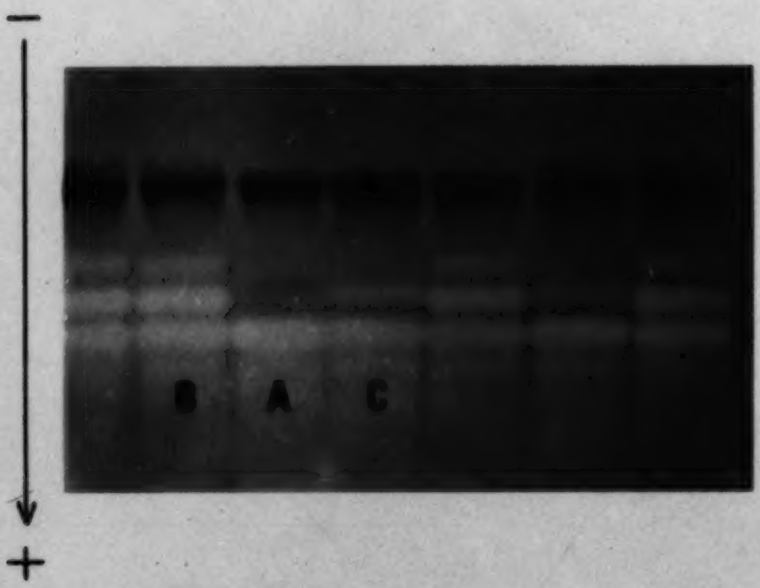


Figure 12. Closeup photograph of the three superoxide dismutase phenotypes seen in Synthetic *Brassica napus*. A - phenotype A; B - phenotype B; C - phenotype C.

Glutamate Dehydrogenase

A single zone of GDH activity appeared on the zymograms, and 7 isozymes could clearly be seen in most cases (Fig. 13).

GDH phenotypes of Groups I and II are shown in Figures 13 and 14, respectively. In the Group II diploids, Chinensis 2N showed no internal variation in band mobility. Individuals of this taxon were quite uniform with respect to relative band intensity, with staining usually greatest in isozymes 3-6. Marrowstem 2N displayed two mobility phenotypes. In the variant Marrow 2-GDH^B isozymes 2-7 were slower than those of Marrow 2-GDH^A, when the bands were compared pairwise. Considerable variation in relative GDH band intensity within a phenotype was present in this taxon, but no classes of variation were evident (see Fig. 15). However, staining appeared to be weighted towards the more cathodal bands.

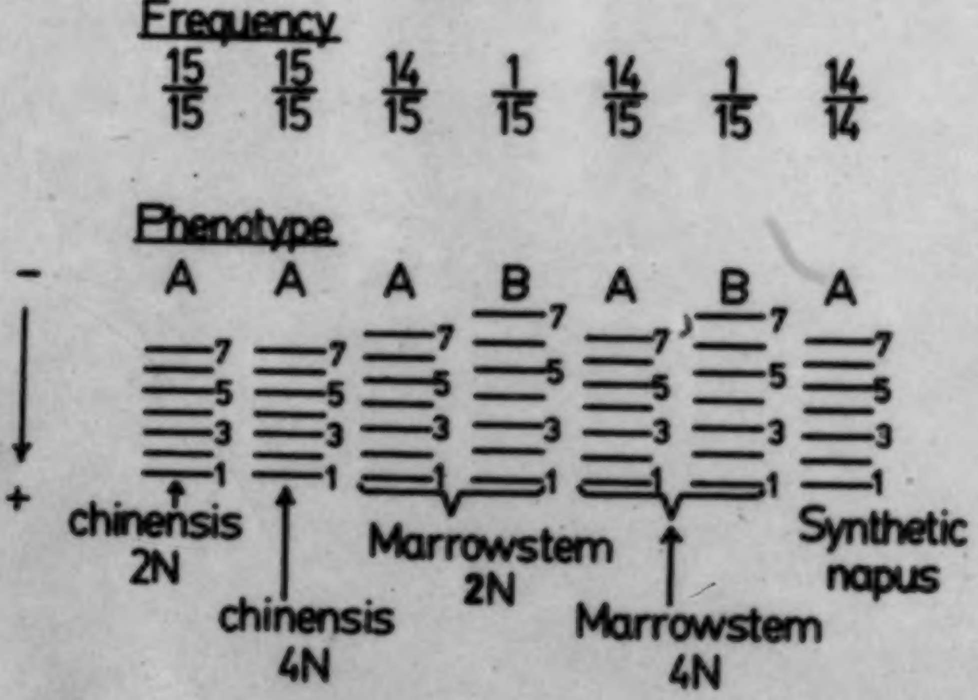
The phenotypes of Marrowstem 2N differed from those of Chinensis 2N. Chin 2-GDH^A and Marrow 2-GDH^A were related in the same fashion as were Marrow 2-GDH^A and Marrow 2-GDH^B.

The phenotypes of the Group II autotetraploids were quite similar to those of their diploid counterparts. Chinensis 4N was indistinguishable from Chinensis 2N. Marrowstem 4N showed the same internal variation in band mobility and relative intensity as Marrowstem 2N.

Synthetic napus exhibited no internal mobility variation. Bands showed the same mobilities as those of Marrow 2-GDH^A and Marrow 4-GDH^A. Bands 2-4 were frequently the most darkly stained of the Syn-GDH^A complement.

Figure 13. Glutamate dehydrogenase zymogram phenotypes of the taxa in Group II. For explanation of format, see Figure 3, p. 37.

Figure 14. Glutamate dehydrogenase zymogram phenotypes of the taxa in Group I. For explanation of format, see Figure 3, p. 37.


Frequency
15/15
15/15
14/15
1/15
14/15
1/15
14/14
Phenotype
A
A
A
B
A
B
A
−
+
7
5
3
1
chinensis 2N
chinensis 4N
Marrowstem 2N
Marrowstem 4N
Synthetic napus

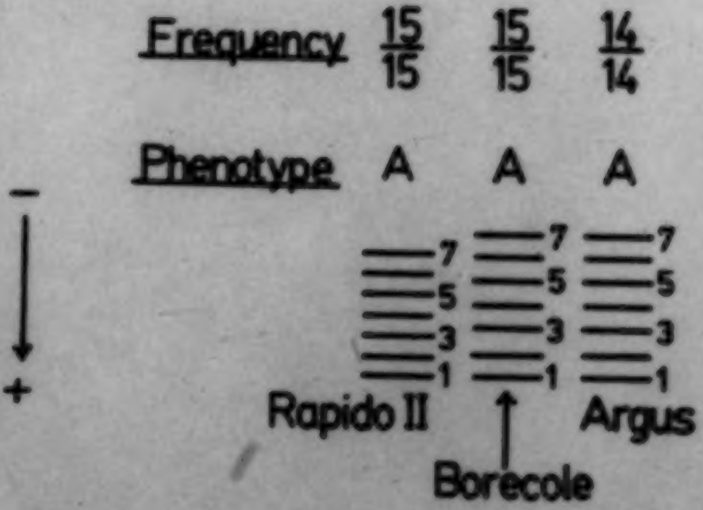
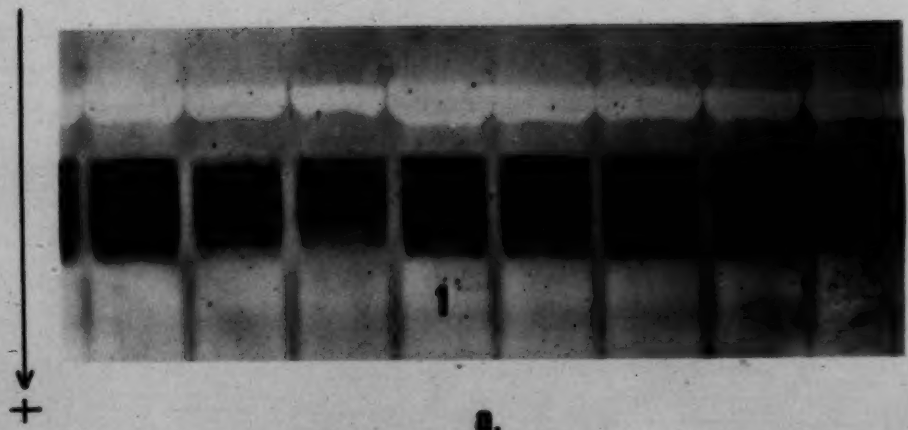

Frequency
15/15
15/15
14/14
Phenotype
A
A
A
−
+
7
5
3
1
Rapido II
Borecole
Argus

Figure 15. Closeup photographs of glutamate dehydrogenase banding patterns in the taxa of Group II.

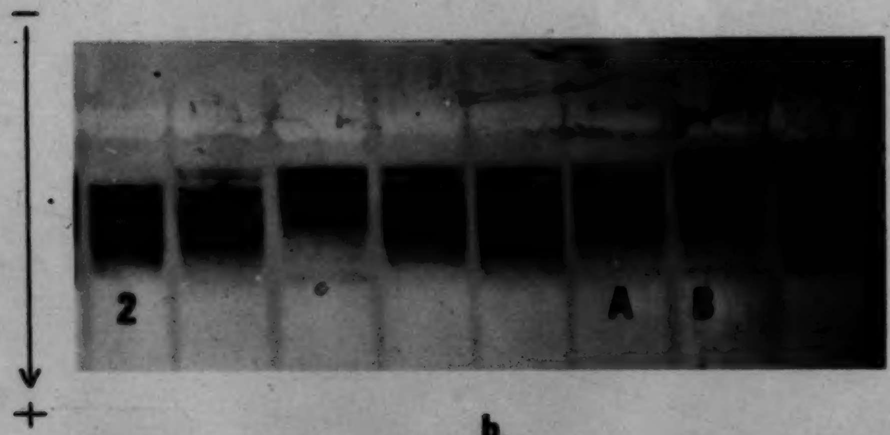
a. Diploid *Brassica campestris* ssp. *chinensis*. 1-Autotetraploid *B. campestris* ssp. *chinensis*.

b. Diploid *B. oleracea* var. *acephala*. A-phenotype A; B-phenotype B.

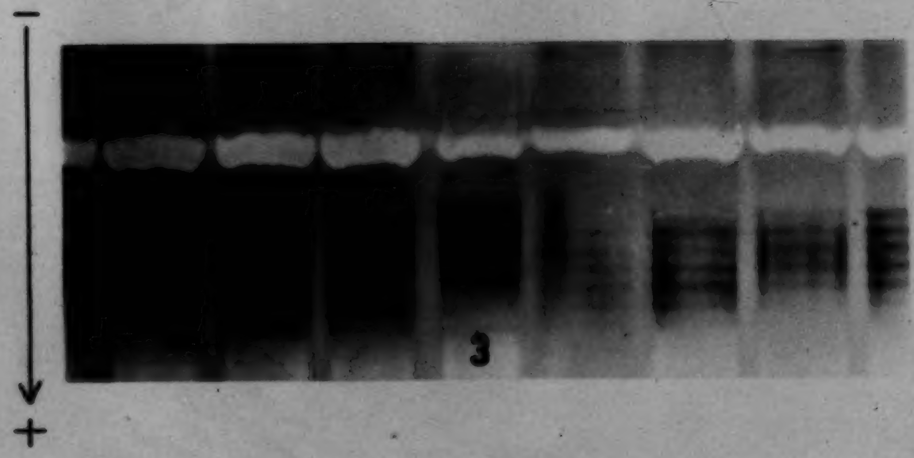
c. Autotetraploid *B. campestris* var. *chinensis*. 3-autotetraploid *B. oleracea* var. *acephala* 'Marrowstem kale', phenotype A.



a.



b.



c.

The GDH zymograms of the Group I taxa bore mutual relationships very similar to those within Group II. Rapido II showed the same lack of internal variation as did Chinensis 2N. Isozymes 2-7 of Rapido II and Borecole differed in mobility in the same pairwise fashion as did these same isozymes in Chin 2-GDH^A and Marrow 2-GDH^A. Borecole was distinct from Marrowstem 2N in that no internal mobility variants were present. Argus resembled Synthetic napus, in that the isozyme mobilities of both alloploids were indistinguishable from those in the most common phenotypes of their *B. oleracea* relatives.

Glutamate-Oxaloacetate Transaminase

GOT was the most complex and variable of the enzyme systems studied on slab gels. The zymograms did not prove tractable to phenotype classification; examples of some GOT gels of three diploid taxa are shown in Figure 16. Identifications of some key features are made in the captions of the Figure, and some salient points with regard to genetic and biochemical interpretation of this system are made in the next Chapter.

Isocitrate Dehydrogenase

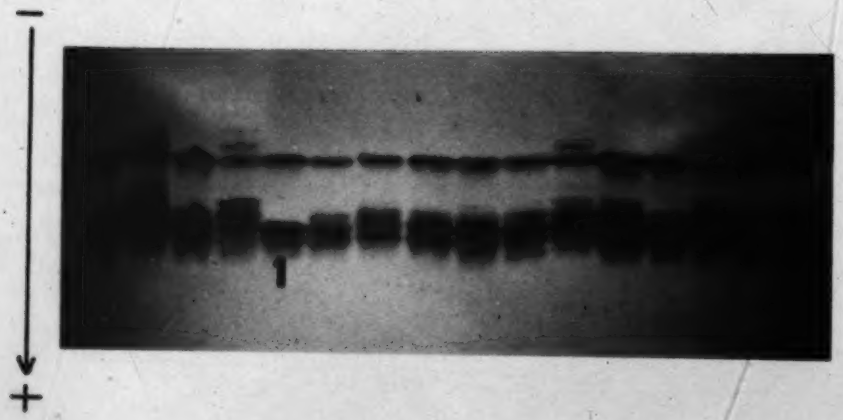
IDH appeared consistently as a single band in all extracts examined. No within-or between-taxon variation was detected. Two gels containing samples from the alloploid taxa and their *a* and *c* genome relatives are shown photographically in Figure 17.

Figure 16. Photographs of glutamate-oxaloacetate transaminase gels of three samples from diploid taxa of *Brassica*.

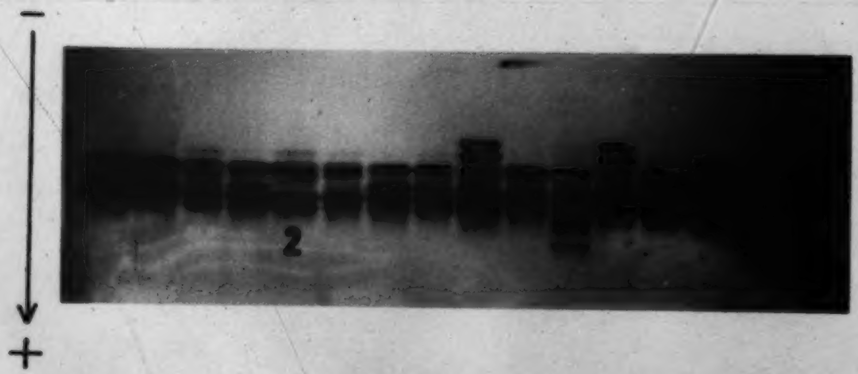
a. *B. oleracea* var. *acephala* 'Borecole'. 1-three-banded phenotype which was the simplest GOT phenotype seen in the study.

b. Diploid *B. campestris* ssp. *chinensis*. 2-simplest phenotype seen in this taxon.

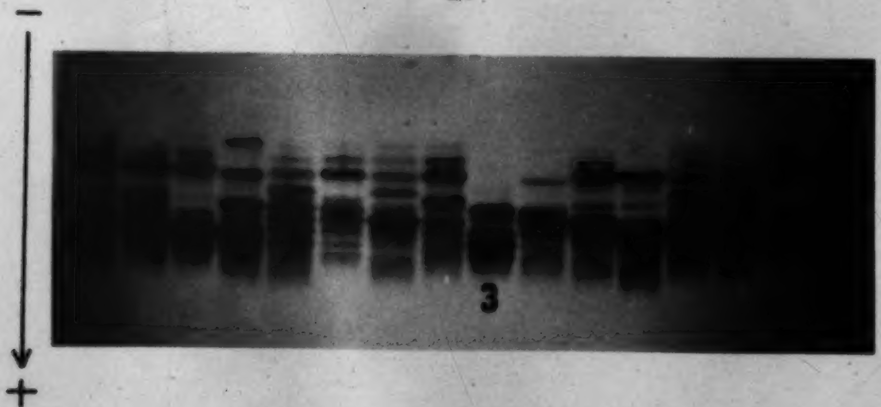
c. Diploid *B. oleracea* var. *acephala* 'Marrowstem kale'. Note extreme variability of banding pattern. 3-Diploid *B. campestris* ssp. *chinensis*.



a.



b.

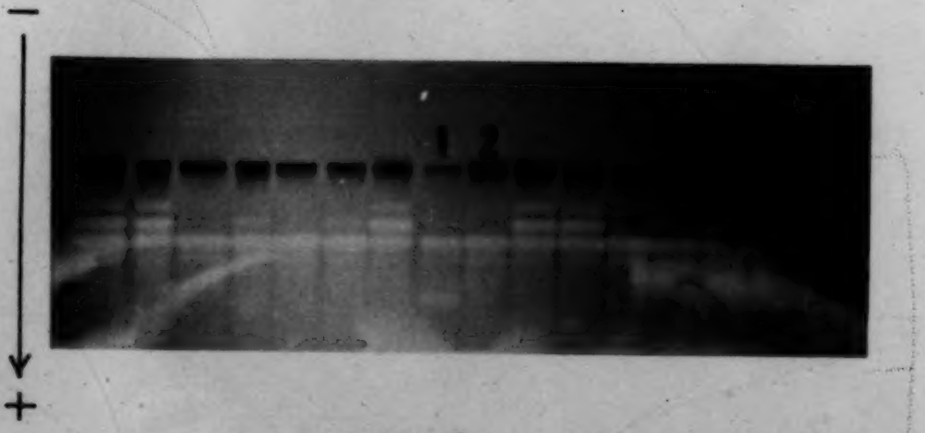


c.

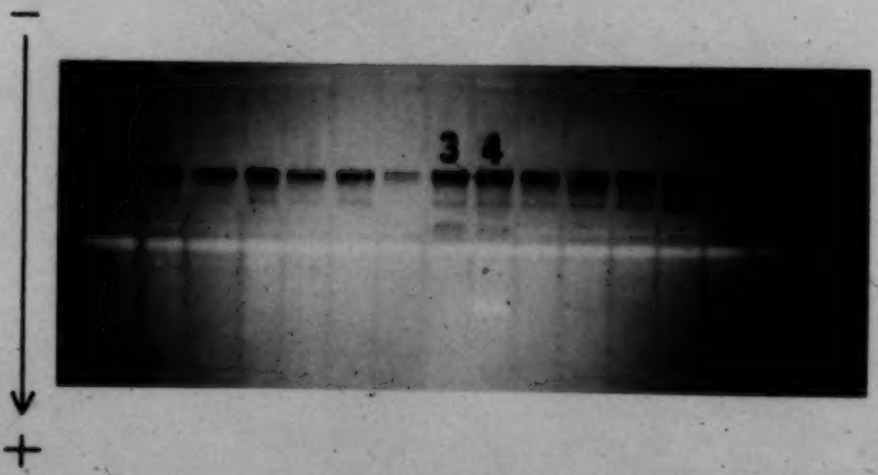
Figure 17. Photographs of isocitrate dehydrogenase gels of two alloploid taxa of *Brassica*. Darkest bands are isocitrate dehydrogenase.

a. Group II. 1 - Autotetraploid *B. campestris* ssp. *chinensis*; 2 - Autotetraploid *B. oleracea* var. *acephala* 'Marrowstem kale'. Remainder of samples are Synthetic *B. napus*.

b. Group I. 3 - *B. campestris* ssp. *oleifera* 'Rapido II'; 4 - *B. oleracea* var. *acephala* 'Borecole'. Remainder of samples are *B. napus* ssp. *oleifera* 'Argus'.
Note identical mobilities of alloploids' IDH bands and those of *a*- and *c*- genome relatives.



a.



b.

DISCUSSION

The main goals of the exposition in this chapter are to analyze the zymogram results just presented in terms of their likely biochemical and genetic bases, and then to extrapolate to their possible meaning in relation to the objectives of this study.

The first section is a brief consideration of the specific *Brassica* material used, with respect to its adequacy for pursuing the stated objectives. Following this, each isozyme system is discussed in turn in terms of (i) biochemical and genetic interpretation of the banding phenotypes observed, and (ii) possible significance of these interpretations for evolutionary processes at the diploid and tetraploid levels in *Brassica*.

In most biosystematic study of established species, many generations have passed since their origin; thus one is unable to compare them to their actual ancestral populations. One is commonly forced to work with present-day descendants of the ancestral populations as "simulated ancestors". This applies equally strongly to alloploids, where even in cases like *Brassica* where the progenitors' affinities can be fairly closely pinpointed, we still must work with extant representatives of these progenitor groups. Thus one goal in setting up comparisons of alloploids with diploid relatives is to find diploid varieties which are minimally different from the actual parental stocks which gave rise to the alloploid.

The allotetraploid member of Group I ('Argus', a European biennial oilseed rape) was chosen to represent established *Brassica napus*. Neither the exact time and place of origin, nor the identities of the parent stocks giving rise to the oilseed rape (*B. napus* ssp. *oleifera*) or the swede (*B. napus* ssp. *rapifera*) is known with any precision. But it is generally considered that oilseed rape arose in northern Europe, as a result of crossing between agricultural varieties of *B. oleracea* and *B. campestris*, at one or more times within the last 400-500 years (Boswell 1949; McNaughton 1976b). Since both turnip (*B. campestris* ssp. *rapifera*) and turnip-rape (*B. campestris* ssp. *oleifera*) were cultivated in Europe at the time, a monotopic and monochronistic origin for *B. napus* involving one or the other of these types as *B. campestris* parent, or a polytopic and/or polychronistic origin involving both of these types, seem equally possible. The plausibility of both alternatives is enhanced by the likelihood that *B. campestris* ssp. *rapifera* arose by artificial selection from a slender-rooted form resembling *B. campestris* ssp. *oleifera* (McNaughton 1976a). However, the results of artificial syntheses of *B. napus* (Olsson 1960b) showed that oilseed- or swede-resembling amphiploids could be produced by crosses involving the corresponding *B. campestris* type. Because of this experimental documentation and perhaps also its greater simplicity, the multiple-origin hypothesis was employed here as a criterion for the choice of the *campestris* member in Group I. Thus Rapido II, a biennial turnip-rape, was chosen to simulate the original donor of the *a* genome to the *B. napus* ancestors of Argus.

The carrier of the *c* genome in the origin of Argus was simulated in Group I by kitchen kale, *B. oleracea* var. *acephala*. This variety appears to have been the earliest of the cabbage and cole group to be cultivated (Thompson 1976). It has been widely grown in Europe since medieval times, and although other candidates (e.g. cabbage, kohlrabi) for the *c* genome parent of oilseed rape exist, kale was chosen as probably resembling the original donor most closely.

The kind of comparison carried out between the members of Group I might be improved by including a wider range of cultivars of all three taxa. This could presumably afford a more reliable picture of the extent of divergence of *B. oleracea, B. campestris* and *B. napus* since the origin of the alloploid lineage.

To provide perspective on possible differences between established and newly-arisen alloploids involving the *a* and *c* genomes, the taxa of Group II were analyzed in parallel with those of Group I. The autotetraploids were samples from exact parental lines which gave rise to the artificial allotetraploid, and so enable a far more rigorous comparison to be carried out between the hybrid and its progenitors. Although the diploid stocks of Group II were not necessarily closely related to their autoploid counterparts, their inclusion was intended to provide points of reference for interpretation of the zymograms. The rigor of the comparisons within Group II could of course have been improved if the diploid stocks used to synthesize the autoploids had been available.

The next five sections are discussions of the results from the five enzyme systems. In addition to the notation and abbreviation outlined in Chapter 3 for isozymes, phenotypes and names of taxa, notation for gene loci and alleles mentioned here is as follows. Structural loci are specified by names written in italics, based on enzymic activities of the proteins for which they code. For example, the locus coding for superoxide dismutase in *Brassica* leaves is called *Sod-1*. Alleles at a locus are signified by a lower-case superscript attached to the locus name; thus for example the fast allele at the *Pgm-1* locus in Chinensis 4N is symbolized by $Pgm\text{-}^{cf}$.

Phosphoglucomutase

Soluble forms of PGM appear in a wide variety of organisms to have a monomeric protein structure. Workers who observe genetically controlled electrophoretic variants of this enzyme routinely report the absence of hybrid isozymes of intermediate mobility in heterozygous individuals (Joshi et al. 1967; McAlpine et al. 1975; Nichols and Ruddle 1973; Torres et al. 1978). In addition, attempts to recover subunits by chemical treatments of the purified native form have mostly been unsuccessful (Ray and Peck 1972). There is however, some evidence that subunits are in fact present (Daugherty et al. 1975; Duckworth and Sanwal 1972).

PGM also appears to be subject to several kinds of variation which are electrophoretically detectable but do not involve differences in the primary amino acid sequence of the protein. These are usually offered as explanations for "epigenetic" (Markert and Whitt 1968) or "secondary" isozyme bands (Shaw 1969). They usually stain more weakly than the main bands, and vary in mobility concomitantly with them. Several structural bases for these secondary bands are considered operative, including oxidation states of sulfhydryl groups (Dawson and Greene 1975), degrees of phosphorylation (Ray and Peck 1972) and conformational isomers (Dawson and Mitchell 1969).

The zone 1 isozymes of PGM in *Brassica* leaves apparently constitute an additional example of a monomerically-behaving enzyme with secondary isozymes. The three phenotypes of *B. campestris* ssp. *pekinensis* (Fig. 4, p. 39) are explained by allelic mobility variation at a single disomic structural locus (*Pgm-1*). Two alleles appear to be present, one (*Pgm-1*^{cs}) coding for a polypeptide with lower mobility than the other (*Pgm-1*^{cf}). Secondary modification would then give two pairs of isozymes, 1+2 and 3+4. Phenotypes pek-PGM^{A} and pek-PGM^{C} then represent homozygotes for the two alleles *Pgm-1*^{cf} and *Pgm-1*^{cs}, while pek-PGM^{B} corresponds to the heterozygous condition.

Applying this schema to the taxa of Group II, no segregation for mobility alleles was detected within Chinensis 2N, Marrowstem 2N or Marrowstem 4N. The difference in mobility between isozymes 1 and 2 of Marrow 2-PGM^{A} and those of Marrow 4-PGM^{A} may indicate the presence of different structural alleles, but the absence in both of these taxa of the pertinent heterozygotes prevents any firm conclusion. The common allele in Chinensis 2N is different from either of those present in the Marrowstem kales. The common allele in Chinensis 2N was provisionally equated with the fast allele of ssp. *pekinensis*, and termed *Pgm-1*^{cf}. The alleles for which the Marrowstem 2N and Marrowstem 4N samples analyzed were monomorphic were termed *Pgm-1*^{m2} and *Pgm-1*^{m4} respectively. In addition, however, Chin 2-PGM^{B} contains no detectable zone 1 PGM activity at all. One explanation for this is that a "null" allele (*Pgm-1*^{n}) exists at the *Pgm-1* locus in this group, and codes for a protein having no visible catalytic activity under the assay conditions employed.Chin 2-PGM^{B} individuals then represent homozygotes for this null allele.

The existence of allozymic proteins having little or no catalytic activity has been demonstrated for a wide variety of enzymes, particularly in *Drosophila* (Bell and MacIntyre 1973; Bewley and Lucchesi 1975; Clark et al. 1978; Collier and MacIntyre 1977; Roberts and Baker 1973). Such mutants may have a wide variety of effects on viability from lethality (Bewley and Lucchesi 1975) to apparent neutrality (Bell and MacIntyre 1973), and the effects may also depend on genetic background (Hughes and Lucchesi 1977). If the Chin 2-PGM^{B} individuals can be considered $Pgm\text{-}1^{n}/Pgm\text{-}1^{n}$ homozygotes, one would also expect to find heterozygous individuals displaying half the zone 1 staining intensity of the normal $Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}$ homozygotes. Such a situation is perhaps visible in zymograms of some individuals (Fig. 5, p. 41). However, the detection of putative heterozygotes for null alleles at a disomic locus would entail the estimation of absolute levels of isozyme activity for which this study is not designed. Therefore, the phenotypic classification of Chinensis 2N does not distinguish between $Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}$ and $Pgm\text{-}1^{cf}/Pgm\text{-}1^{n}$ individuals.

In Chinensis 4N also, segregation is displayed for the two alleles $Pgm\text{-}1^{cf}$ and $Pgm\text{-}1^{cs}$. In an autotetraploid, two alleles at a locus imply five possible genotypes instead of the three possible in a diploid. Considered in this light, phenotypes Chin 4-PGM^{A}, Chin 4-PGM^{B} and Chin 4-PGM^{C} appear to represent quadriplex ($Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}$), triplex ($Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}/Pgm\text{-}1^{cs}$) and duplex ($Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}/Pgm\text{-}1^{cs}/Pgm\text{-}1^{cs}$) genotypes respectively.

Proceeding to Synthetic napus, all four *Pgm-1* phenotypes found in this taxon are readily explained on the basis of genetic contributions from parental autotetraploids. Syn-PGM^{A} would thus correspond to a $Pgm\text{-}1^{cf}/Pgm\text{-}1^{cf}$ constitution for the *campestris* locus plus $Pgm\text{-}1^{m4}/Pgm\text{-}1^{m4}$ at the *oleracea* locus in the amphiploid. Similarly, Syn-PGM^{B} is $Pgm\text{-}1^{cf}/Pgm\text{-}1^{cs} + Pgm\text{-}1^{m4}/Pgm\text{-}1^{m4}$. In this case, the overlap in mobility between isozyme 3 of Chin 4-PGM^{B} and isozyme 2 of Marrow 4-PGM^{A} explains the presence of 5 bands instead of the expected 6 (See Fig. 7). Syn-PGM^{C} and Syn-PGM^{D} can be explained on the basis of a null allele similar to the one presumed for Chinensis 2N, which was present in the Chinensis 4N stock but was either not present or not detectable in the sample of individuals analyzed. Syn-PGM^{C} individuals can then be seen as $Pgm\text{-}1^{n}/Pgm\text{-}1^{n} + Pgm\text{-}1^{m4}/Pgm\text{-}1^{m4}$, and Syn-PGM^{D} individuals as $Pgm\text{-}1^{n}/Pgm\text{-}1^{cs} + Pgm\text{-}1^{m4}/Pgm\text{-}1^{m4}$.

In Group I the zymogram phenotypes can be understood on a very similar basis. Rapido II appears to be segregating for two mobility alleles at Pgm-1, with mobilities such that isozyme 3 of the slow allele ($Pgm\text{-}1^{s}$) overlaps with isozyme 2 of the fast allele ($Pgm\text{-}1^{r}$). Thus, the heterozygote $Pgm\text{-}1^{r}/Pgm\text{-}1^{s}$ displays the phenotype RII-PGM^{B}, with only 3 of the expected 4 bands. Borecole also contained some internal variation in phenotype, but as with Marrowstem 2N and Marrowstem 4N, the apparent absence of heterozygotes precluded confident interpretation. Bore-PGM^{A} was provisionally called homozygous for $Pgm\text{-}1^{b}$.

The uncertainty of close ancestral relationship between Argus, Rapido II and Borecole renders misleading any literal explanation of Argus phenotypes in terms of genotypes of Rapido II and Borecole. However, it seems safe to say that Arg-PGM^{A} can be understood in terms of genotypes very similar to $Pgm\text{-}1^{s}/Pgm\text{-}1^{s} + Pgm\text{-}1^{b}/Pgm\text{-}1^{b}$. Isozyme 3 of "RII-PGM^{C}" apparently overlaps with isozyme 2 of "Bore-PGM^{A}", giving 3 bands instead of the expected 4. That this overlap is likely to occur in amphiploids carrying *Pgm-1*^{a} and *Pgm-1*^{b} is apparent from examination of RII-PGM^{C} and Bore-PGM^{A} individuals run in parallel (Fig. 8). Arg-PGM^{B} individuals can be "explained" similarly as having *Pgm-1*^{r}/*Pgm-1*^{a} + *Pgm-1*^{b}/*Pgm-1*^{b} genotypes, and Arg-PGM^{C} individuals as having *Pgm-1*^{r}/*Pgm-1*^{r} + *Pgm-1*^{b}/*Pgm-1*^{b} genotypes. In these cases, additional mobility overlaps are assumed between: (i) isozyme 2 of "RII-PGM^{B}" or "RII-PGM^{C}" and isozyme 2 of "Bore-PGM^{A}", and (ii) isozyme 1 of "RII-PGM^{B}" or "RII-PGM^{C}" and isozyme 1 of "Bore-PGM^{A}". The zymogram phenotypes of true RII-PGM^{B}, RII-PGM^{C} and Bore-PGM^{A} individuals run in parallel, and the increased relative intensities of isozymes 2 and 3 of Arg-PGM^{B} and Arg-PGM^{C}, support the notion of their internal heterogeneity (Fig. 8, 9).

How do the biochemical and genetic interpretations just outlined for zone 1 PGM phenotypes relate to the stated objectives of the study? First, no direct evidence for multisomic inheritance was found in any of the diploids. The segregant phenotypes of all four *B. campestris* taxa examined agree with a hypothesis of disomy for the chromosome carrying the *Pgm-1* locus in the diploids. The observation of apparent tetrasomic segregation patterns in Chinensis 4N strengthens this hypothesis. Since no clear-cut segregants were seen in *B. oleracea*, the level of duplication of the corresponding chromosomes in this species remains open to question.

The second stated objective of this study was to trace the expression in *Brassica* alloploids of genome contributions of their diploid progenitors. Apparently *Pgm-1* of both parents is expressed in the alloploids of Group I and Group II, and the mobilities of all isozymes in the hybrids are derivable from allelic forms seen in the diploids. The *aacc* taxa appear to be permanently heterozygous for *Pgm-1* alleles derived from both parents. The age of the alloploid made no difference in this regard, as both Argus and Synthetic napus showed this conservative relationship with their diploid relatives. If phosphoglucomutase is in fact monomeric, no novel heteromeric isozymes were to be expected, and none were found.

Ohno (1970) proposed that selective pressures on duplicated gene loci would be altered, since previously deleterious mutations affecting gene function may be tolerated in redundant copies. Although Ohno and others(Bailey and Wilson 1970; Freeling and Schwartz 1973; Gottlieb 1977; Markert *et al.* 1975) have stressed the potential of this mechanism for structural and functional divergence of loci, other workers have emphasized the tendency for redundant gene copies to be "silenced" by accumulation of structural and/or regulatory mutations (Engel *et al.* 1975; Ferris and Whitt 1977a, b; Gomez-Campo *et al.* 1978).

Granting that allopolyploidy brings together divergent genomes and thus may not always represent "true duplication" for a given locus, it seems evident that at least a portion of the loci of an alloploid would show redundancy of function. One might therefore expect to find evidence in the zymogram phenotypes of alloploids for divergence or silencing of portions of the progenitors' genomes, as well as persistent codominant expression of other portions. Also, the likelihood of patterns which diverge from co-expression of both parental contributions would seem to increase with the age of the alloploid. *Pgm-1* in leaves of Argus and

Synthetic napus in this study seems not to have changed from a purely codominant pattern of expression. This may be due to the relatively recent origin of both alloploids, to some selective advantage accruing from the permanently heterozygous condition, or to a combination of both factors.

Superoxide Dismutase

SOD has been identified as an enzyme which catalyzes the reaction $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ (McCord and Fridovich 1969). It has attracted considerable attention from both biochemists and geneticists, and has now been shown to be widely distributed among aerobic organisms. In eukaryotes it exists in two distinct forms which are apparently of different evolutionary origin. One active form is mitochondrial and exists as a Mn - containing tetramer; the other is found in the cytosol and is a Cu - and Zn - containing dimer. Evidence for the dimeric structure of the cytosolic form has come from genetic (electrophoretic) and from biochemical (purification) studies. "Hybrid isozymes" in individuals heterozygous for electrophoretically variant forms of SOD have been observed in organisms as phylogenetically diverse as *Helix pomatia* (Wahren and Tegelström 1973), *Drosophila melanogaster* (Jelnes 1971), *Salmo gairdneri* (Cederbaum and Yoshida 1972), *Dipodomys* (Johnson and Selander 1971), and man (Beckman 1973). Published reports of electrophoretic hybrid forms of SOD in plants are infrequent, but biochemical studies have indicated that the purified enzymes from *Pisum sativum* (Sawada *et al.* 1972), *Spinacia oleracea* (Asada *et al.* 1973) and *Triticum aestivum* (Beauchamp and Fridovich 1973) are also dimeric.

The assumption that the active form of soluble SOD in *Brassica* leaves is dimeric appears to be a safe one. On this basis, zone 1 SOD phenotypes in this material may be interpreted as follows.

None of the *B. campestris* taxa showed any variation in zone 1 phenotype as defined in Chapter 3. Although this prohibits estimation of structural locus dosage in Chinensis 2N, Chinensis 4N and Rapido II, the two zone 1 isozymes are provisionally considered to be coded by the *Sod-1* locus in all taxa. This locus is electrophoretically homozygous in Chinensis 2N and 4N. Isozymes 1 and 2 were judged both to be products of the same allele ($Sod\text{-}1^c$) at the *Sod-1* locus, since they occurred together in every sample and their intensities varied concomitantly. A similar electrophoretic pattern has been reported for human SOD by Beckman and Beckman (1975). Since the most common alleles in all the other Group II taxa had the same mobility, these were also named $Sod\text{-}1^c$.

In interpreting the variant SOD phenotypes seen in Marrowstem 2N, Marrowstem 4N, Synthetic napus, Borecole and Argus, the following schema was useful. A single-locus heterozygote A/B is assumed to produce subunits α and β which combine to form catalytically active dimers. If the subunits combine at random three kinds of dimers - $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ will result. The proportion of each type of dimer in the mixture is given by the expression,

$$(p + q)^2 = p^2 + 2pq + q^2 \qquad (1)$$

where p and q represent the relative quantities of subunits α and β which are produced. If one assumes further that α and β are produced in equal amounts and that the dimers $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ are equally active catalytically, $p = q = \frac{1}{2}$ and the zymogram phenotype of the heterozygote should display 3 bands in the intensity ratio 1:2:1.

The marked asymmetry of Marrow 2-SOD^B thus may have a biochemical or a genetic basis. The biochemical explanation suggests that α and β have different effects on the catalytic efficiency of the dimers into which they are incorporated, so that under the staining conditions employed the order of activities of the dimer types is $\alpha\alpha > \alpha\beta > \beta\beta$. Thus, although the proportions of dimer types may still be 1:2:1, their unequal activities per molecule produce an asymmetric zymogram profile. The genetic explanation assumes that α and β have negligibly different effects on activity, but are produced in differing amounts based on gene dosage or expression. This would involve a change in p and q, and an asymmetric ratio of quantities of $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ would result.

The SOD data from any one taxon alone did not permit distinction between these two possible explanations. However, comparison of results from all 8 taxa provided a framework for preliminary interpretation. First, Marrowstem 4N as a recently arisen synthetic autotetraploid is almost certainly at least tetrasomic for the chromosome carrying the putative *Sod-1* locus. Therefore, in a simplex heterozygote for a variant β subunit which differs from the α subunit only in electrophoretic mobility, $p = \frac{3}{4}$ and $q = \frac{1}{4}$ in expression (1). This gives a ratio of $\alpha\alpha$, $\alpha\beta$ and $\beta\beta$ subunits of 9:6:1, which approximates the band intensity ratio seen by visual inspection in Marrow 4-SOD^B. Thus a genetic explanation of Marrow 4-SOD^B plants as simplex heterozygotes for a slow allele $Sod\text{-}1^{m4}$ apparently fits the data.

Since if one accepts this genetic explanation for Marrow 4-SOD^B *Sod-1* is presumably therefore disomic in Marrowstem 2N, a biochemical explanation seems more likely for Marrow 2-SOD^B. The observation of a variant in Borecole which gives the symmetric phenotype Bore-SOD^{B} would also appear to support a hypothesis of disomy for *Sod-1* in the *c* genome. Electrophoretic variants of human SOD with diminished activity have been reported by Beckman *et al.* (1973). The variant alleles in Marrowstem 2N and Borecole were provisionally named $Sod\text{-}1^{m2}$ and $Sod\text{-}1^{b}$ respectively.

The two variant phenotypes of Synthetic napus are of interest, since they can be explained genetically on the basis of different dosages of *Sod-1* in its *B. campestris* and *B. oleracea* parents. Almost certainly the *Sod-1* locus from each parent is present in at least disomic dosage. In the case of disomy in both parental genomes, one would expect a heterozygote at one of the two homoeologous loci to behave much as a simplex heterozygote in an autotetraploid, presumably giving an asymmetric phenotype resembling Marrow 4-SOD^{B}. One would also expect to observe only one class of asymmetric phenotypes with band intensities weighted towards isozyme 2.

Such is not the case in Synthetic napus; two different asymmetric phenotypes are seen. If one parent (possibly Chinensis 4N) were tetrasomic for the chromosome carrying *Sod-1*, p and q in expression (1) would be $\frac{5}{6}$ and $\frac{1}{6}$ respectively for a $Sod\text{-}1^{c}/Sod\text{-}1^{m4}$ heterozygote at the *c* genome *Sod-1* locus, and $\frac{4}{6}$ and $\frac{2}{6}$ respectively for a $Sod\text{-}1^{m4}/Sod\text{-}1^{m4}$ homozygote at this locus. The expected band ratios for these two cases are 25:10:1 and 4:4:1, which may be considered to correspond by visual estimate to Syn-SOD^{C} and Syn-SOD^{B} respectively.

The results for Argus may also bear on this hypothesis. The phenotypes of the sample analyzed from this taxon are uniformly asymmetric.

All individuals show two bands (isozymes 3 and 4) with mobilities similar to those seen in the *B. oleracea* taxa but with substantially lesser intensity. The constant presence of isozymes 3 and 4 suggests homozygosity for an allele similar to *Sod-1*^{b} at the *c* genome *Sod-1* locus in Argus, and the asymmetry is consistent with multisomy of the homoeologous *a* genome locus.

To summarize the discussion of SOD thus far in light of the first objective of this study, it appears that analysis of this enzyme has some potential for attacking questions of structural relationship between the *a* and *c* genomes of *Brassica*. Insufficient evidence is available as yet to distinguish conclusively between genetic and biochemical explanations for the zymogram phenotypes observed, but some possible correspondences with Röbbelen's (1960) genome formulas have been detected.

Because of the identity of mobility for isozymes 1 and 2 of the diploid and autoploid taxa, it was not possible to identify the separate gene products of the *a* and *c* genomes in every individual of Synthetic napus or of Argus. Isozymes 3 and 4 of Syn-SOD^B, Syn-SOD^C and Arg-SOD^A are the only evidence that *Sod-1* of the *c* genome is expressed in the alloploids. As implied in the discussion above, *Sod-1* of the *a* genome is presumed to be expressed, but direct evidence is lacking.

Allelic variation detected at the *Sod-1* locus in the alloploids is fully accounted for by alleles occurring in the diploid and autoploid taxa. Fixed heterozygosity for divergent parental alleles probably described the situation in Argus, but this condition was not electrophoretically detectable in Synthetic napus. No novel isozymes were observed in the zymograms. However, if both parental *Sod-1* loci are expressed, isozyme 3 in the alloploids is expected to consist of intergenomic heterodimers as well as dimers composed only of *c* genome subunits. Such intergenomic molecular hybridization is expected to occur freely with SOD in *Brassica* alloploids; this is supported by the results of Tegelström (1975), who was able to perform *in vitro* hybridization of SOD subunits from animal species belonging to different phyla.

Glutamate Dehydrogenase

GDH of plants is a large protein of molecular weight ca. 200,000 (Fawole 1977; Pahlich 1972), the structure of which is very poorly understood. However, one fairly consistent feature of the enzyme is its isozyme profile in disc electrophoresis. A 7 band pattern very similar to the one seen here in *Brassica* leaves has been detected in *Vicia faba* (Thurman *et al.* 1965; Fawole 1977), *Pisum sativum* (Pahlich 1972), *Ricinus communis* (Lee 1973), *Zea mays* (Yue 1969) and *Phaseolus mungo* (Yue 1969). A 6 banded pattern was seen in *Arachis hypogaea* (Yue 1969).

Using the technique of Hedrick and Smith (1968), wherein disc gel densities are varied in order to resolve contributions of molecular shape and charge to mobility, Pahlich (1972) obtained evidence that the GDH isozymes of *Pisum sativum* differ in molecular charge. Thurman *et al.* (1965) and Lee (1973) established by elution-and-re-electrophoresis experiments that the isozymes composing the individual GDH bands in *Vicia faba* and *Ricinus communis* are chemically stable. In *Ricinus communis*, the faster and slower bands have different relative staining intensities in different tissues, and only the two fastest bands stained when $NADP^+$ instead of NAD^+ was supplied as cofactor. These results suggest that the isozymes of GDH seen in *Brassica* and other species (i) are not artifacts of electrophoresis, and (ii) are of some physiological significance, perhaps playing differing roles in catabolism and anabolism of glutamate (Lee 1973).

The GDH phenotypes of *Brassica* leaf extracts did not yield any hypotheses for the mode of structural gene control of the enzyme. However, one feature of the patterns may be of genetic significance. Firstly, it is pointed out that in electrophoretic studies of enzymes structural variants are generally expressed in a genetically codominant fashion, i.e. heterozygotes are distinguishable from either homozygote by the simultaneous presence of variant and "wild type" bands. Secondly, the variant phenotypes Marrow 2-GDH^B and Marrow 4-GDH^B were present in low frequency in the population samples of these taxa. Therefore, if we assume that these low-frequency phenotypes are based on possession of a low-frequency mutant allele which is probably present in the hemizygous state, then from the absence of isozymes 1-7 of Marrow 2-GDH^A and Marrow 4-GDH^A in these variant individuals it would appear that the putative mutant gene is expressed as a genetic dominant.[1]

Although this shema obviously requires further experimental ratification, if accurate it may be an example of genetic variation for aspects of protein structure distinct from primary amino acid sequence. Recent work on xanthine dehydrogenase (XDH) in *Drosophila* has shown that heritable variations in electrophoretic behaviour of enzymes can sometimes be mapped to gene loci which are topographically remote from the enzyme structural locus but interact epistatically with it (Finnerty *et al.* 1979). As Johnson (1979) points out, there is no *a priori* reason why mutants at such epistatically interacting loci should not be expressed as genetic dominants, giving a single gel band of altered mobility instead of the classic heterozygote pattern of two or more bands. If, as is considered likely for XDH, post-translational modification of the enzyme is the basis for epistatic interaction, a wide variety of modes of inheritance would seem plausible at this stage of knowledge, since the nature of such secondary alterations in protein structure is not well understood (Uy and Wold 1977). Whether or not an analogous explanation exists for the GDH variation observed in *Brassica* is a question reserved for future work.

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[1] The alternative is that Marrow 2-GDH^B and Marrow 4-GDH^B represent low frequency homozygotes for the putative mutant gene, but the only altered consequence of this would be that the "wild type" gene is genetically dominant instead.

In reference to the objectives outlined in the Introduction, since gene loci could not be reliably identified in the GDH system no information relevant to the structural relationships of the *a* and *c* genomes was gained. With respect to parental genome expression in the alloploids, the *c* genome seems to show the same kind of "dominance" relationship with respect to the *a* genome as the putative genetic variants controlling Marrow 2-GDH^B and Marrow 4-GDH^B show towards the common alleles in these taxa. This is concluded from the absence of bands resembling those of the *B. campestris* taxa in mobility.

No "novel" bands were seen in either alloploid; nor were any hybrid bands evident. No differences whatsoever were seen between Argus and Synthetic napus. The genetic relationships which are responsible for the GDH phenotypes of *Brassica* alloploids therefore appear not to have changed over the period since the origin of the line leading to Argus.

Glutamate-Oxaloacetate Transaminase

On the basis of hybrid bands seen in electrophoresis, soluble forms of GOT have frequently been suggested to exist in active form in plants as dimers (Gottlieb 1973; Hart and Langston 1977; Scandalios *et al.* 1975; Torres *et al.* 1978). It is also observed in a wide variety of plant species to be controlled by 2 or more structural gene loci (Gottlieb 1973; Levin 1977; Scandalios *et al.* 1975; Torres *et al.* 1978).

The overall complexity of genetic control for GOT in *Brassica* is suggested by the fact that no individual in the entire slab gel study had a zymogram profile with fewer than 3 bands. The 3-banded phenotype was seen in Borecole (Fig. 16a), and suggests that the number of loci controlling soluble GOT in the *c* genome is at least 3. In addition, considerable allelic segregation is probably taking place, as illustrated by the extreme phenotypic variability of Marrowstem 2N (Fig. 16c).

The difficulty in interpreting GOT phenotypes as connected with alleles at specific structural loci is apparently due to the very similar migration rates of the different gene products. This seems to cause extensive mobility overlap between variant molecules at different loci and greatly complicates the use of allelic variants to frame genetic hypotheses. No attempt is made here to frame such hypotheses, but with its multiple gene loci, substantial stores of allelic variation and dimeric structure, this enzyme system offers considerable potential for further study. It offers a rich supply of genetic markers for probing structural relationships of the *a* and *c* genomes and parental locus expression in the alloploids. But in addition, the evolutionary roles of the multiple loci and structural allele variation themselves may prove interesting. For example, one might ask how effective different alleles are in hybrid isozyme realization, or if intergenic as well as intragenic heterodimers are formed. Furthermore, how have these subunit interactions evolved?

Future work on these questions should first emphasize development of electrophoretic techniques capable of separating products of the various GOT loci.

Isocitrate Dehydrogenase

No evidence concerning the genetic control or subunit structure of IDH in *Brassica* was forthcoming from the results of this study. Expression of the specific IDH structural genes of the *a* and *c* genomes accordingly cannot be traced in the alloploids, although it is noted that no novel isozymes were detected. The enzyme does not deviate in this respect from the other systems which were investigated.

Table 4 (page 81) summarizes the allelic variation found at the structural loci *Pgm-1* and *Sod-1* in this study.

Table 4. Summary of alleles of enzyme structural loci identified in *Brassica*. For definitions of taxon names, see page 32. For definitions of Groups I and II, see Table 2, page 16.

Locus	Taxon	Alleles
Sod-1	Group I	
	Rapido II	*Sod-1*^{c}
	Borecole	*Sod-1*^{c}, *Sod-1*^{b}
	Argus	*Sod-1*^{c}, *Sod-1*^{b}
	Group II	
	Chinensis 2N	*Sod-1*^{c}
	Chinensis 4N	*Sod-1*^{c}
	Marrowstem 2N	*Sod-1*^{c}, *Sod-1*^{m2}
	Marrowstem 4N	*Sod-1*^{c}, *Sod-1*^{m4}
	Synthetic napus	*Sod-1*^{c}, *Sod-1*^{m4}
Pgm-1	Group I	
	Rapido II	*Pgm-1*^{r}, *Pgm-1*^{s}
	Borecole	*Pgm-1*^{b}
	Argus	*Pgm-1*^{r}, *Pgm-1*^{s}, *Pgm-1*^{b}
	Group II	
	Chinensis 2N	*Pgm-1*^{cf}, *Pgm-1*^{n}
	Chinensis 4N	*Pgm-1*^{cf}, *Pgm-1*^{cs}
	Marrowstem 2N	*Pgm-1*^{m2}
	Marrowstem 4N	*Pgm-1*^{m4}
	Synthetic napus	*Pgm-1*^{cf}, *Pgm-1*^{cs}, *Pgm-1*^{n}, *Pgm-1*^{m4}

CONCLUSIONS

The primary objective in the work reported here was to generate working hypotheses of structural gene control for five selected enzyme systems of an alloploid complex in *Brassica*. Questions surrounding (i) genome structure in diploids, and (ii) parental genome expression in allotetraploids in the species complex could then be addressed, using these genetic hypotheses as a framework.

Two enzyme systems of the five investigated, phosphoglucomutase and superoxide dismutase, yielded results which lent themselves to the identification of individual structural loci. The considerable non-overlap between the *a* and *c* genomes in the electrophoretic mobilities of isozymes coded by the PGM structural locus was very useful in tracing genome expression in the *aacc* alloploids. Both genomes were clearly expressed, and allelic variation at the progenitors' loci explained completely the zymogram phenotype variation in the allotetraploids. This suggests that other enzyme systems containing electrophoretically detectable intergenomic allele differentiation will also be useful in studying genome expression in these *Brassica* alloploids. Another interesting result from this enzyme was that autotetraploids of *B. campestris* ssp. *chinensis* displayed clear zymogram evidence for tetrasomic segregation of alleles at the *Pgm-1* locus. This strongly suggests that the electrophoretic techniques used here are applicable in at least some enzyme systems to studies of gene dosage and chromosome duplication in the *a* and *c* genomes of *Brassica*. Although no such explicit zymogram evidence of tetrasomic segregation was found in any of the diploid taxa studied here, an expanded search with this and other enzymes may yield the desired information.

The identification of an SOD structural locus in this material led to some preliminary evidence for differential chromosome duplication in the *a* and *c* genomes. The observation of asymmetric banding intensities in the heterozygotes for variant alleles at this locus suggested that the locus is present in greater than disomic dosage in the *a* genome. However, to a sizeable degree the products of the homoeologous *a* and *c* genome *Sod-1* loci were indistinguishable in electrophoretic mobility. This prevented the clear observation of parental SOD gene expression in the alloploids.

The feasibility, once gene loci have been identified, of using zymogram data to study genome structure and expression in *Brassica* has thus been clearly demonstrated with phosphoglucomutase and superoxide dismutase. Nine other enzyme systems (Table 3, p. 30) have also been shown in this study to be analyzable using the same plant material and electrophoretic techniques. Extension of the questions and procedures to these other enzymes would therefore be most worthwhile.

The results obtained with the remaining three enzymes in the group of five were not reducible to terms of alleles and loci. Nevertheless, glutamate-oxaloacetate transaminase holds great potential if electrophoretic separation techniques can be developed to effectively resolve the products of the three or more gene loci judged to be present. The apparently large amount of allelic variation in this system promises to give a useful array of gene markers; furthermore, the evolutionary role of the inter- and intralocus variation itself could be studied. Glutamate dehydrogenase afforded a possible example of multilocus control of enzyme structure. Future work might develop from these observations as starting points.

Turning to a different issue, a brief discussion will ensue concerning the connection between chemotaxonomy on the one hand and the genetic approach to electrophoresis on the other. "Chemotaxonomy" is defined as the use of chemical characters to shed light on evolutionary relationships and classification. The "genetic approach to electrophoresis" is here limited to the analysis of protein and enzyme banding patterns on gels in terms of alleles and loci.

Electrophoretic data have frequently been employed, as chemical characters, to help solve taxononmic problems. A commonly used approach involves the comparison of gel banding patterns by a variety of techniques intended to define the degree of similarity or difference between two taxonomic groups, without any attempt to determine the genetic bases of the bands (Booth and Richards 1978; Houts and Hillebrand 1976). This non-genetic type of analysis is in fairly widespread use, even though proteins are considered by chemotaxonomists to represent more fundamental characters due to their being gene products only a few steps removed from the genetic and phylogenetic information in the DNA itself (Smith 1976).

The potential chemotaxonomic value of even the very simple kind of genetic information yielded by the present study of *Brassica* is considerable. For instance, a commonly encountered problem with electrophoretic data is in ensuring reproducible biochemical technique and standardized biological material. If one can derive a simple genetic interpretation for observed variation, explanations attributing the variation to technical artifact or uncontrolled physiological variables might be more easily discarded. Another taxonomic use of genetic information relates to the assignment of taxonomic weight to different types of band variation. As an example, if presence of a new band were ascribable to the presence of a new gene locus, it would presumably be given more taxonomic weight than if allelic variation at a single locus were responsible. Finally, an advantage to expressing comparative electrophoretic data in terms of alleles and loci is that the quantity of genetic information being used in the comparison can be precisely specified. This is not usually possible with other types of phenotypic information. Gottlieb (1977) discusses this point at greater length.

In summary, the results of the present work demonstrate the usefulness of electrophoresis in the study of some aspects of genetic relationships and evolutionary change in *Brassica*. In addition, they illustrate the relative ease with which genetic hypotheses can be formulated from zymogram data alone. The use of such an approach is recommended as a preliminary step in chemotaxonomic comparisons using electrophoretic data.

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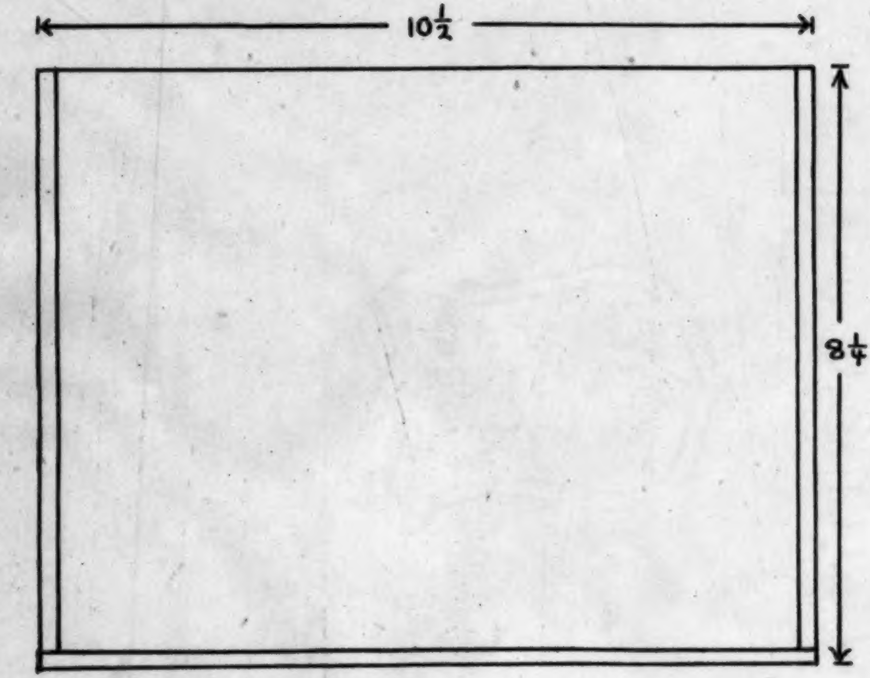
APPENDIX 1

The illustrations on the following five pages show the structural dimensions of the apparatus constructed for parallel co-electrophoresis in slab gels.

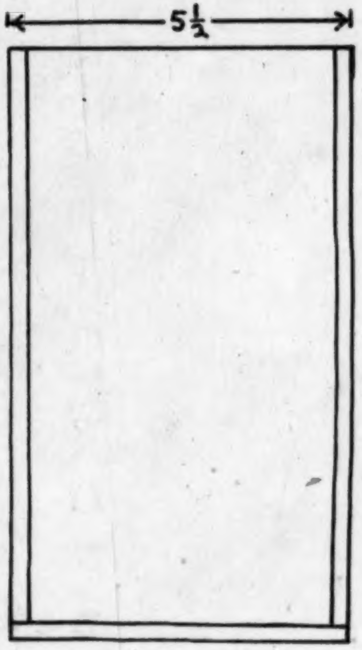
APPENDIX 2

Photosynthetically active radiation, measured at bench level with an integrating sensor, was 185 - 210 $\mu Em^{-2}sec^{-2}$ in the growth room used.

OUTER TANK



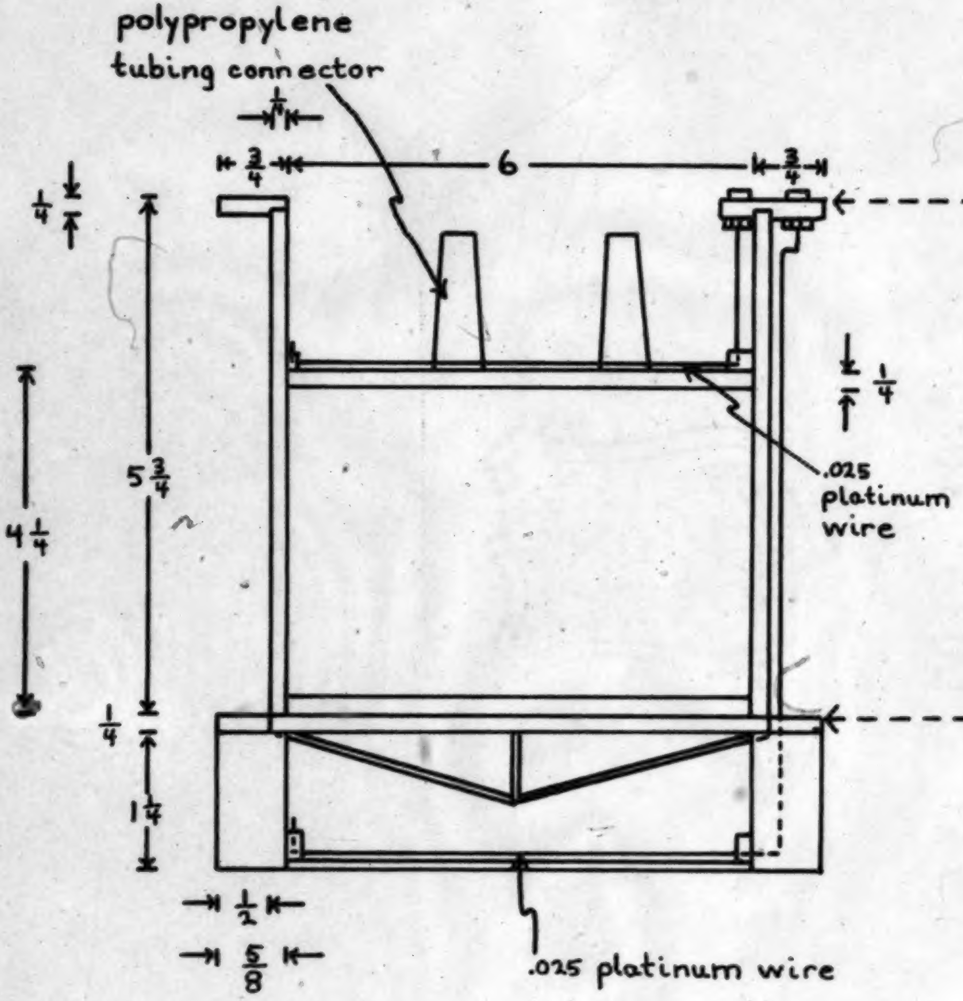
SIDE VIEW



END VIEW

Materials : ¼" acrylic

INNER CHAMBER (SIDE VIEW)


polypropylene tubing connector
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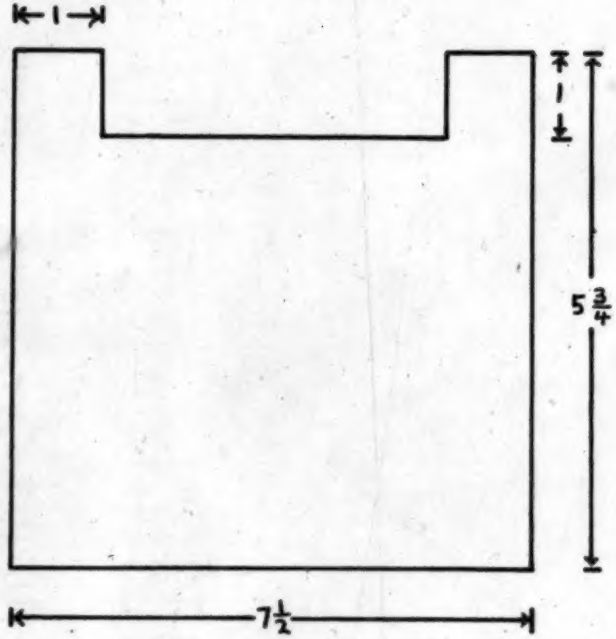
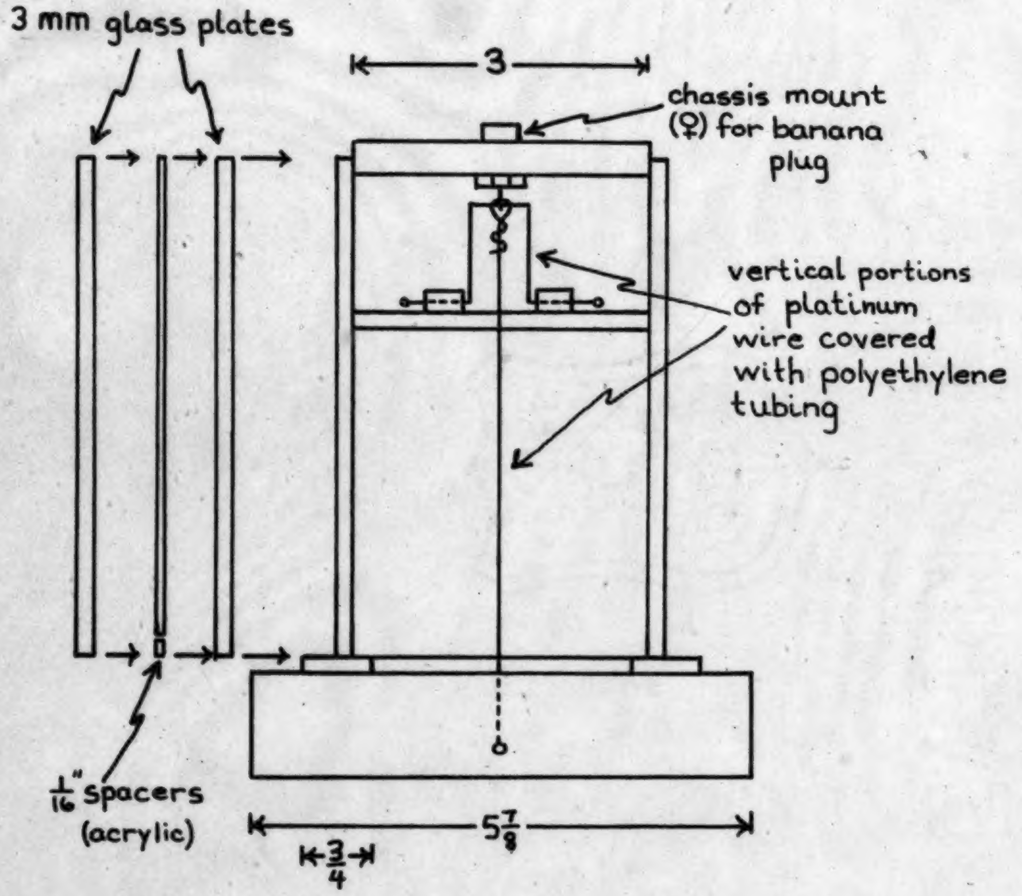
.025 platinum wire
.025 platinum wire



FACE PLATE

INNER CHAMBER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chassis mount
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plug
vertical portions
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tubing
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(acrylic)
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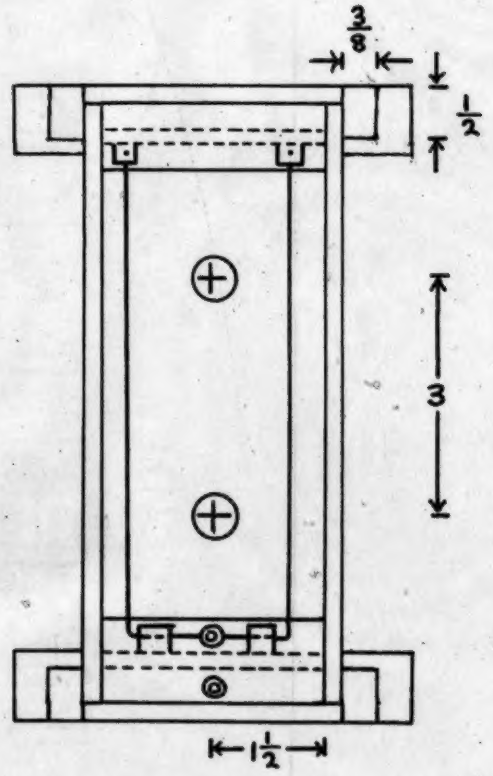
END VIEW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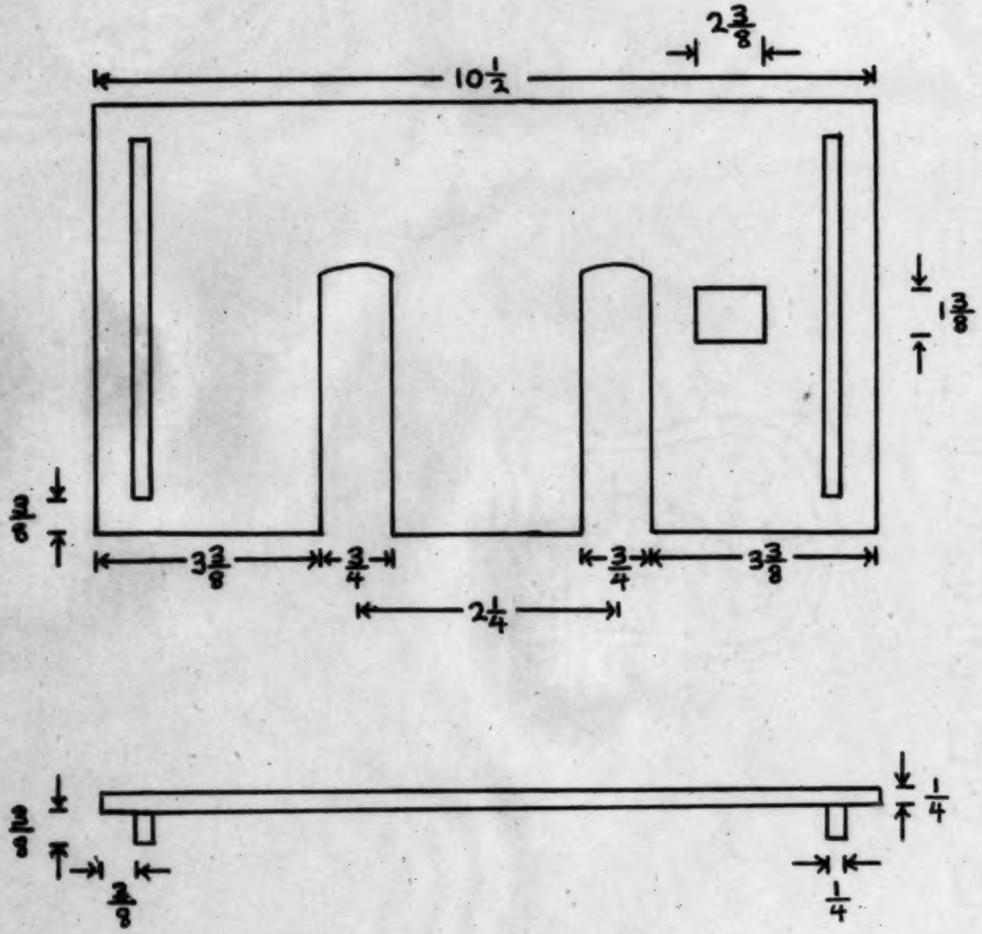
TOP VIEW

TANK LID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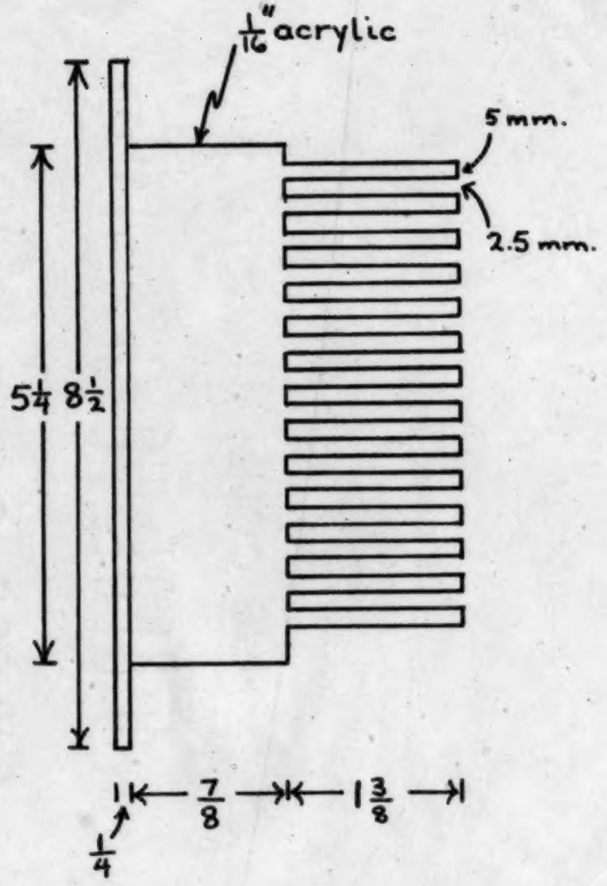
$\frac{1}{4}$

GEL COMB


$\frac{1}{16}$" acrylic
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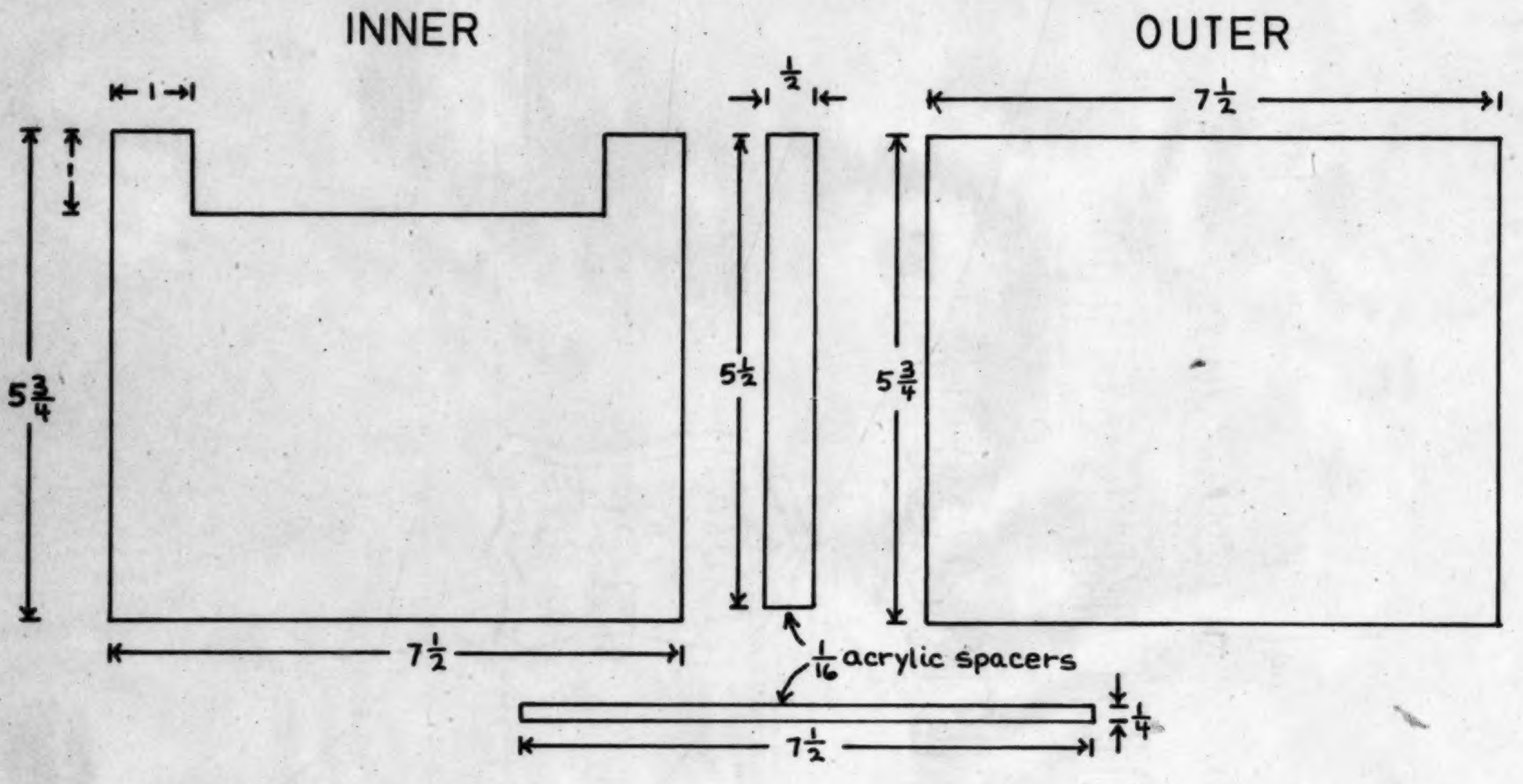
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5 3/4
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1/16 acrylic spacers
1/4
7 1/2

GEL PLATES (3mm. plate glass)

Sources of Materials for Slab Gel Apparatus

Material	Source
Glass plates	Mainland Crystal Glass Co., 6424 103 St., Edmonton, Alberta
Platinum wire	Johnson Matthey and Mallory Ltd., Toronto, Ontario
Acrylic plastic Electrical connections Polyethylene tubing Nylon screws	Stores Division, University of Alberta, Edmonton, Alberta

APPENDIX 3

Notes on Electrophoresis Technique

Several excellent publications are available which treat of various types of electrophoretic techniques (see references at the end of this Appendix). In addition, the following outline may prove useful at least as far as the procedure is applied to *Brassica* leaf enzymes.

A. Extraction of Enzymes

In the extraction of enzymes directly from leaf material into an aqueous medium, it is important that the medium be buffered. An unbuffered extract of many plant tissues tends to be quite acidic, and such extremes of pH often affect enzyme activity adversely. An extraction medium matching the stacking gel buffer in pH (6.7) and chloride concentration (0.6 M) is recommended in discontinuous gel systems such as the one used here.

Another problem charactecteristic of plant tissues is the near-ubiquitous presence of phenolic compounds. These interact with proteins in two major ways. One way is by non-specific hydrogen bonding of the -OH groups of tannins to the =O of the peptide bond, leading to the precipitation of insoluble tannin-protein complexes. The other way is by enzymic or non-enzymic oxidation of diphenolic species to form highly reactive quinones, which may then react chemically with proteins and cause loss of activity. These two problems were approached in the present study by the inclusion in the extraction buffer of (i) Polyvinylpyrrolidone, a large molecular weight compound which is considered an effective alternative complexing agent for phenolics, and (ii) Dithiothreitol, a potent reducing agent, to slow the interaction of phenolics with oxygen. In addition, a pH slightly on the acid side of neutrality is considered to be the least favorable for quinone formation.

The extraction buffer also included EDTA, a cation-complexing agent, to help prevent deleterious interactions of proteins with metals, and several cofactors (NAD^+, $NADP^+$, and pyridoxal phosphate) which have been reported to stabilize dehydrogenases and transaminases.

The buffer contained 10% sucrose to increase sample density and facilitate layering onto the gel. Care must be taken when using slab gels not to use too high a concentration of sucrose in the extraction buffer. Too great a disparity in osmotic potential between sample and gel may disturb the orderly movement of proteins into the stacking phase, resulting in "streaking" and loss of resolution. A further caution is that this extraction buffer should be adjusted to final pH only after all of the solutes have been allowed to come to equilibrium in solution.

Large ratios of buffer volume are considered desirable for plant enzymes. This tends to dilute small molecules like phenolics and to increase the efficiency of buffering. Furthermore, in a good many enzyme systems, low activity of the initial extract is compensated by the concentration effect during stacking in multiphasic gel systems. Large sample volumes (up to 100 microlitres) are commonly used with no negative effect of the volume *per se* on quality of resolution.

When ground glass homogenizers are used to crush leaf material, the buffer should be added first and the finely-divided sample added second. This improves intimate contact between the cell contents and the medium. Also, care should be taken to avoid excessive "foaming" of the extract, caused by too-vigorous motion of the pestle within the homogenizer barrel. Disruption of tertiary and/or quaternary structure of proteins, with attendant loss of enzyme activity, may result from the altered surface tension conditions in thin films such as bubbles.

B. Casting of Gels

The advantages of multiphasic electrophoresis are only fully exploited in a well-cast gel. Not only does resolution seem to be improved when a sample is electrophoresed through a geometrically regular series of gel phases, but reprodicibilty may be improved as well. The following procedure has been found to produce disc and slab gels of high quality.

Disc Gels

1. Seal the ends of the glass tubes with Parafilm, and slip a rubber cap on over this, The Parafilm insures an even surface on the bottom of the gel. Place the sealed tubes in a gel casting stand or in the grommets of the upper buffer reservoir of the Buchler apparatus.
2. Add the required amounts of Small Pore (1) and Small Pore (2) to separate vacuum-tolerant containers with a boiling chip in each. For the size of glass tubes used in this project, 1.5 ml of

final gel solution per tube is sufficient. Degas the solutions by evacuating the containers on the line vacuum supply. Bubble formation can be accelerated by gently swirling or tapping the containers on a padded surface.

3. Add the appropriate amount of Small Pore (2) to the Small Pore (1) container and stir briefly with a glass rod. Work quickly after this stage, as the mixture will polymerize in 10-20 minutes.
4. Add the polymerization mixture to the sealed glass tubes with a syringe or a disposable pipet, taking care to introduce no air bubbles.
5. Layer water on the solution surface, using a syring with a small- bore needle and a short length of polyethylene tubing attached. The gel solution and water must be at the same temperature, otherwise convection will disrupt the interface between the two phases.
6. Allow to polymerize at room temperature.
7. When the running gel has polymerized, shake out excess water and rinse the surface twice with spacer gel solution. Then pour the spacer gels and layer water as before. The spacer gel solution may be mixed up to several hours in advance if stored in the dark; 8 ml of solution will be enough for about 12 gels.
8. Place the fluorescent lamp directly above the tubes, as close as possible to the spacer gel solution. Illuminate until the yellow solution has become opaque and white throughout. The gels are now ready for sample application.

Slab Gels

1. Apply a little vaseline to the plastic cooling plate of the inner

chamber. When the notched glass plate is then laid on this surface, the upper buffer reservoir should be effectively sealed from the lower except for the space between the glass plates which is occupied by the gel.

2. With the acrylic spacers in between, lay the outer rectangular glass plate on top of the inner notched plate (see diagram, page 110). Clamp the plates to the chamber with metal spring clips along the sides of the resulting glass "sandwich". Assemble the second pair of glass plates in the same way, on the opposite side of the inner chamber.
3. Drip a boling agar solution (1.5% w/v) along the sides of the glass plates, to seal them. This is most effectively done by removing the clips one at a time and replacing them as agar is applied to that section of the plate edges. Then apply the agar to the bottom edges of the plates and clamp them. To complete the seal, tilt the inner chamber endwise at an angle of 45° and drip a little agar solution between the plates with a pipet. This will form a triangular plug at each of the lower corners of the space between the glass plates.
4. Insert a gel comb between each pair of plates to aid in judging the required volume of solution to pour the running gels.
5. Mix the volume of running gel solution required for one slab gel (about 24 ml, depending on the depth of spacer gel desired), after degassing as described above. Introduce the solution into the space between the plates with a syringe having polyethylene tubing attached, running the solution gently down one side spacer. Layer water onto the surface as with a disc gel, again using a syringe with attached tubing. Apply the water at one upper corner of the gel, and then at

the other. Repeat the pouring and layering process for the other gel.

6. Allow to polymerize at room temperature.
7. Remove excess water and rinse the running gel surface twice with spacer gel solution. With the well-forming comb in place, introduce the spacer gel solution and polymerize under fluorescent light. 8 ml of spacer gel solution is required for each gel. Note: The spacer gel should be allowed to sit for at least 1 hour after polymerization before attempting to remove the well-forming combs. Otherwise, residual unpolymerized gel solution may cause enough suction upon comb removal that the interstitial colums of gel separating the slots will distort or break.
8. Remove the combs carefully, and replace the spring clips with the plastic clamps. With the bottom pair of clamps on each gel only slightly tightened, gently push the spacer on the lower gel edge out from between the glass plates, using e.g. one prong of a pair of blunt forceps. Tighten the clamps. The gels are now ready for sample application.

C. Sample Application and Apparatus Assembly

Application of small, precise volumes of extract is conveniently accomplished with a 100-microliter syringe. In layering the aliquot under the cathode buffer, it helps to place the tip of the syringe needle as close as possible to the spacer gel surface. This minimizes mixing with the cathode buffer.

To apply samples to slab gels and begin electrophoresis:

1. Fill the sample slots with cathode buffer.

2. Apply the samples.
3. Fill the inner chamber half-full with water. This prevents the chamber from floating when immersed in the anode buffer.
4. Lower the inner chamber into the outer tank, which should contain about 3 liters of anode buffer. Hold the inner chamber on an endwise slant when doing this, to prevent bubble collection in the space between the glass plates at the bottom of the gel.
5. Add about 500 ml of cathode buffer to the upper reservoir.
6. Place the lid on the tank, connect the electrodes and begin the separation, with the entire apparatus in the refrigerator.

D. Slab Gel Removal

1. When the separation is completed, remove the inner chamber from the tank and pour out the water and cathode buffer. The anode buffer may be re-used up to 5 times.
2. Remove the plastic clamps and side spacers from one gel.
3. With the chamber on its side, gently prise the outer plate off. The gel should remain on the lower plate for maximum ease of handling.
4. With a kithchen spatula, lift the gel from the lower plate and transfer it to the appropriate staining solution.

Suggestions for Further Reading on Electrophoresis Theory and Technique

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